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EDITORS
W. J. CROZIER JOHN H. NORTHROP
W. J. V. OSTERHOUT

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THE NATURE OF THE FACTORS WHICH DETERMINE THE SEQUENCE OF GROWTH-CYCLES AND ITS RELATIONSHIP TO THE DIFFERENTIATION OF TISSUES.

By T. BRAILSFORD ROBERTSON.

(From the Darling Laboratories of Biochemistry and General Physiology, University of Adelaide, Adelaide, Australia.)

(Accepted for publication, May 25, 1928.)

The Origin of Asymmetry in Growth-Cycles.

In analysing the growth of the white mouse into its component processes[1] it was found that the first and most prolonged growth-cycle, which extends throughout the growing period in these animals, is of the asymmetrical type which is defined by the equation:—

$$\log \frac{x + b}{A - x} = k(t - t_1) \qquad (1)$$

where A represents the maximum growth attainable, k the velocity-constant of the growth-process, t_1 is the moment of maximum growth-velocity and b is a constant the essential significance of which is that growth-velocity is already appreciable when x is zero. It also has the effect of rendering the two halves of the cycle on either side of the moment of maximum growth-velocity, unequal in slope and amplitude.

The remaining growth-cycles (the second and third) which are of comparatively brief duration, are of the symmetrical type which is represented by the equation:—

$$\log \frac{x}{A - x} = K(t - t_1) \qquad (2)$$

in which the constants have the same significance as above, but growth-velocity is zero when x is zero and the two halves of the growth-cycle, on either side of the moment of maximum growth-velocity, are equal in slope and amplitude.

[1] Robertson, T. B., *J. Gen. Physiol.*, 1925–28, viii, 463.

The applicability of these equations to the growth of mice extends to these animals the rule which has previously been found to obtain in the growth of a variety of other animals[2] and of plants as well,[3] the rule, namely, that the growth of an organism and, frequently, of parts of an organism as well, is an autocatalysed process, by which nothing more is necessarily implied than that *growth is a process of such a nature that it facilitates its own occurrence.* This follows immediately from an inspection of the differential forms of the above equations which may be written:—

$$\text{Symmetrical type:—}\ \frac{dx}{dt} = kx(A - x) \tag{3}$$

$$\text{Asymmetrical type:—}\ \frac{dx}{dt} = k(x + b)\ (a - x) \tag{4}$$

each of which may be regarded as an equation of the monomolecular form, in which the velocity-constant increases in the first case in direct proportion to the product, that is, to the amount of growth achieved, in the second case in proportion to the product (= growth achieved) plus some quantity (= b) which remains unaltered throughout the growth of the animal and has the effect of ensuring that at the beginning of the cycle (which, in the mouse, corresponds with the beginning of gestation) the velocity of growth is considerably greater than that which we would otherwise anticipate from the initial weight of the fertilized ovum.

So far as these facts go, and they may now be considered to have been very thoroughly established in a diversity of forms, they may appear to prove nothing beyond the fact, familiar enough when not formulated in mathematical terms, that growth facilitates its own occurrence. They reveal in addition, however, the fact that this facilitation is quantitative and proportionate to the amount of growing material, and also the fact that growth is limited by some factor ($=A$) which may either represent some fixed amount of material which

[2] For literature see Robertson, T. B., The Chemical basis of growth and senescence, Monographs in experimental biology, Philadelphia, 1923; also Brody, S. J., *J. Gen. Physiol.*, 1925–28, viii, 233.

[3] For literature see West, C., *Sc. Progr.*, 1921–22, xvi, 382.

is consumed during growth or, on the contrary, some accumulation of material which inhibits growth by its mass-action, as a chemical equilibrium is inhibited by the accumulation and mass-action of the products of the forward reaction. The equations reveal nothing whatever regarding the nature of these facilitating and limiting factors in growth which, so far as this information alone is concerned may be physical conditions just as conceivably as they may be chemical substances, provided only that they fulfill the quantitative relation of proportionality to growth achieved which is involved in the applicability of the equations. We are entitled to state, however, as an empirical law of growth, that *the time relations of the growth-process in animals and plants are similar to those which pertain in autocatalysed chemical reactions.* Mere mathematical analysis of the growth-curve, taken by itself, will not lead us further in the interpretation of the actual mechanism of the growth-process. To enable this we must seek additional information of a more directly chemical description. Evidence of this type is afforded by Le Breton and Schaeffer's estimations of the changes in the nucleo-cytoplasmic ratio which accompany the growth of animals.[4]

It has frequently been observed in the growth of plants that the autocatalysis is of the asymmetrical type which is defined by equations (1) and (4).[5] In animals this asymmetry has been less frequently observed because its effect upon the contour of the growth-curve is greatest at the beginning of growth (when b is large in comparison with x), and the beginning of growth, in the higher animals, is generally inaccessible to accurate measurement because it occurs *in utero.* Nevertheless, one may clearly infer that asymmetry occurs by exterpolating the autocatalytic formulæ of the symmetrical type, which are found to fit the post-natal growth of the animals, to the beginning of gestation. It is then found that the calculated weight at the beginning of gestation is of appreciable magnitude instead of being virtually

[4] Le Breton, E., and Schaeffer, G., Variations biochimiques du rapport nucleoplasmatique au cours du developpement embryonnaire, Travaux de l'institut de Physiologie, Faculté de Médecine de Strasbourg, Paris, 1923.

[5] Enriques, P., *Biol. Zentr.*, 1909, xxix, 331. Prescott, J. A., *Sultanic Agric. Soc. Bull., No. 13,* Cairo, 1924. Brody, S. J., *J. Gen. Physiol.*, 1925–28, viii, 233.

zero[6] and this magnitude affords an approximate measure of the constant b in the asymmetrical formula which truly defines the growth of the animal. We may therefore conclude that asymmetry of the growth-curve is the rule rather than the exception, and we find, in the mouse at all events, that where the growth-curve in any cycle appears to be symmetrical the cycle is one of very brief duration, covering only a fraction, and that a comparatively late one, of the total growing period.

The asymmetrical equation is equivalent to a symmetrical formula in which the velocity-"constant" varies in proportion to the ratio:—

$$\frac{x + b}{x}$$

which, of course, approaches unity as x (= growth achieved) increases. The asymmetry of the curve of growth implies, therefore, that the velocity-"constant" in the autocatalytic formula does not maintain its initial value but falls off as growth proceeds and in the proportion indicated. The relative values of the velocity-"constants" at various stages of the growth of the animal or plant may therefore be computed from the equation:

$$\frac{k^1}{k} = \frac{x + b}{x}$$

where x is the weight-increment from the moment of fertilization which is attributable to the particular (asymmetrical) cycle under consideration.

If we plot the relative values of k^1, thus computed, against the total weights of the animals, we obtain a curve which at first falls steeply and later more slowly, approaching the asymptotic value of $k^1 = k$. If, on the same diagram, we plot the relative magnitudes of the corresponding nucleo-cytoplasmic ratios, as estimated chemically by Le Breton and Schaeffer[7] we find that the two curves are almost precisely parallel in contour, so that, if laid over one another, they almost coincide.[8] From this we may conclude, without any assump-

[6] Robertson.[1]

[7] Le Breton and Schaeffer.[4]

[8] Robertson.[1]

tion as to causation, that, as an empirical fact, k^1 is proportional to $\frac{Nc}{Cy}$ where Nc is the mass of nucleic aid contained within the nuclei of all the tissues of the mouse, taken collectively, and Cy is the mass of protein, probably exclusively extra-nuclear.[9]

Although this relationship has as yet been discovered only in the white mouse, because the alteration of nucleo-cytoplasmic ratios with growth has not yet been ascertained in any other organism of which the growth-curve has been accurately defined, yet it is exceedingly improbable that the correspondence of the two curves is accidental, or that any relationship so simple and fundamental is peculiar to one or a few species of animals. It may therefore be justifiable to generalise this result and to state that *the velocity-constant in any autocatalytic cycle of growth is proportional to the nucleo-cytoplasmic ratio.* In what follows we shall assume this rule to be generally applicable and inquire into the nature of the consequences which must necessarily flow therefrom. The fundamental character of these consequences and their close affiliation to the conclusions which are indicated by modern discoveries in genetics, contribute to justify our assumption that proportionality of growth-velocity to the nucleo-cytoplasmic ratio is of general application and not a specific peculiarity of the mouse.

Before proceeding to the consideration of these consequences, however, it must be pointed out that the absence of quantitatively appreciable asymmetry in the later and briefer growth-cycles of the white mouse does not constitute any exception to the rule which we have assumed, because the alteration of the nucleo-cytoplasmic ratio at the relatively late period of growth at which they occur, and for the duration of the periods which these cycles cover, is too small to affect their velocity-constants to a sufficient extent to distort the symmetry of the autocatalytic curve. Such growth-cycles are presumably asymmetrical, but the degree of asymmetry is so small as to fall within the limits of experimental error.

The Sequence of Growth-Cycles.

It has been shown in the communication referred to above[10] that any number of autocatalytic growth-processes, occurring simultaneously

[9] Abderhalden, E., and Kashiwado, T., *Z. physiol. Chem.*, 1912, lxxxi, 287.

[10] Robertson.[1]

within the tissues of an organism, must, *provided that they share a common catalyser*, fuse into a single autocatalytic curve of weight-accretion. "The several processes, in other words, must lose their identity in the combined result."[11] The experimental fact, now demonstrated in many species of animals, is that there are two or three distinguishable autocatalytic cycles which contribute to the total growth of the animal without losing their identity in so doing. Only one conclusion can be drawn from this fact, namely, that there exist autocatalytic growth-processes or groups of such processes within the animal and probably, of course, in plants also,[12] which do not share a common catalyser. Such processes may occur within the same cells, or, more probably, in different cells and, in this latter case, we must assume that the several growth-cycles which contribute to the total growth represent the growth of different tissues which share a common catalyser with tissue cells of the same kind, but not with cells of other kinds of which the growth is defined by other and separable autocatalytic curves.

This being the case one would expect to find the velocity constant of autocatalysis varying in proportion to changes in the cells which participate in that cycle, but not, as we have found, in proportion to changes occurring in the tissues of the organism as a whole. If the velocity-constant of autocatalysis were truly dependent upon the nucleo-cytoplasmic ratio, we would expect it to vary in proportion to the change of this ratio occurring in the cells participating in the growth expressed by that cycle, but not to the change in other cells which participate in other cycles. But the estimations of Le Breton and Schaeffer were made upon the whole animal, not upon particular tissues, so that we are driven to infer that the several groups of cells, of which the growth constitutes the different cycles which are distinguishable in the total growth of the animal, nevertheless control or modify each other's growth in some other fashion than by the sharing of a common catalyser.

Only two alternative explanations of this phenomenon appear to be

[11] Robertson.[1]

[12] Thus the growth of a fruit (Robertson, T. B., *Arch. Entwcklngsmechn. Organ.*, 1908, xxv, 581), or seed (Prescott[5]) represents an autocatalytic process clearly distinguishable from the growth-curve of the plant as a whole.

open to us. Either we must assume that tissues which do not participate in a given growth-cycle nevertheless control it by some "action at a distance" or unintelligible "entelechy" which is not the sharing of a common catalyser, or we must assume that the nucleo-cytoplasmic ratios in all the tissues not participating in the first cycle remain stationary until, in consequence of the growth of the cells contributing to the first cycle, their nucleo-cytoplasmic ratios fall to the average value which characterises cells of the second cycle, which then, and not until then, begin to grow, so that the nucleo-cytoplasmic ratios of both groups of cells thereafter fall together in the same proportion. Then, when the cells which contribute the first and second cycles have undergone diminution of their nucleo-cytoplasmic ratios sufficient to bring the ratio down to the level characteristic of the third group of cells, they, in their turn, begin to grow, so that the ratios of all of the cells fall thereafter in proportion to one another.[13]

The ratio $\frac{Nc}{Cy}$ is, in fact:—

$$\frac{Nc_1 + Nc_2 + Nc_3}{Cy_1 + Cy_2 + Cy_3}$$

where Nc_1, Nc_2 and Nc_3 are the nuclear masses in the cells which participate in the first, second and third growth-cycles respectively, and Cy_1, Cy_2 and Cy_3 are the corresponding cytoplasmic masses. The hypothesis just outlined indicates a means, and, so far as our knowledge extends, the most probable means of ensuring that the fall of nucleo-cytoplasmic ratios with growth shall be uniform in all the cycles, whether one, two or three cycles are participating in the total growth of the animals during the period under consideration, so that, as we have experimentally ascertained, it must follow that the fall of nucleo-cytoplasmic ratios in the animal as a whole correctly represents the fall in any of the groups of cells which are at the moment participating in the growth of the animal.

[13] There may, of course, be sundry subsidiary groups of which the growth is inappreciable when merged in the total growth of the animal, so that their growth does not lead to experimentally perceptible fluctuation of the total growth-curve. Such minute subsidiary cycles would be lost to view in the mathematical analysis of the growth of the entire animal.

There are, however, certain implications involved in this hypothesis which merit most careful consideration. Thus, the true nucleo-cytoplasmic ratio from the beginning of the first cycle to the beginning of the second, is not $\frac{Nc_1}{Cy_1}$ but $\frac{Nc_1 + N}{Cy_1 + C}$, where $N = Nc_2 + Nc_3$ and $C = Cy_2 + Cy_3$. The velocity-"constant" of autocatalysis of growth, during this period, has been found to be proportional to the nucleo-cytoplasmic ratio *in the animal as a whole*, that is, to $\frac{Nc_1 + N}{Cy_1 + C}$, and this cannot be equal to $\frac{Nc_1}{Cy_1}$ unless $\frac{N}{C}$ is also equal to this ratio, which it could not be unless it varied proportionately with it, that is, unless growth of the second or third groups of cells were occurring, or unless, alternatively, N and C were both so small as to be negligible in comparison with Nc_1 and Cy_1. Now nearly all of the cells which will ultimately constitute the adult animal have already been produced at birth, and the great majority of them considerably before this.[14] Practically all of the great increase of weight which occurs after birth is due to the increase in the size of cells, only an insignificant fraction of the total growth being attributable, at this stage, to the production of new cells. The data which we are discussing, and the conclusions derived therefrom, apply almost entirely to the growth of pre-existing cells. Clearly, therefore, cells which have not yet undergone growth since their production during embryonic development will constitute, during the earlier stages of growth, a large proportion of the total *number* of cells in the animal, but a very small proportion of the total *weight* of the animal. Hence, since the total growth of the animal prior to the initiation of the second cycle is quantitatively attributable to the first cycle[15] the contribution to the weight of the animal due to cells of the second and third groups must be a negligible proportion of

[14] For example, see Ellis, R. S., *J. Comp. Neurol.*, 1920, xxxii, 1 (Purkinje cells). Macallum, J. B., *Bull. Johns Hopkins Hosp.*, 1898, ix, 208 (Muscle cells).

[15] The "linear increment" which, in mice, is superadded to the growth due to the three autocatalytic cycles, is of inappreciable magnitude at this stage.

the total,[16] so that N and C must, as the second of the above alternatives requires, be negligible in comparison with Nc_1 and Cy_1.[17]

It follows, in other words, that during the period that growth remains as yet solely attributable to the first cycle, $\frac{Nc_1 + Nc_2 + Nc_3}{Cy_1 + Cy_2 + Cy_3}$ remains substantially equal to $\frac{Nc_1}{Cy_1}$ because Nc_2, Nc_3, Cy_2 and Cy_3 are, as yet, negligibly small quantities, growth having not yet occurred in the corresponding cell groups. Subsequently to the initiation of the *second* cycle, $\frac{Nc_1 + Nc_2 + Nc_3}{Cy_1 + Cy_2 + Cy_3}$ remains equal to $\frac{Nc_1}{Cy_1}$ because Nc_3 and Cy_3 are, as before, negligibly small quantitatives, and Nc_2 and Cy_2, however large they may become, do not begin to increase until $\frac{Nc_1}{Cy_1}$ falls to the initial value of the ratio $\frac{Nc_2}{Cy_2}$ and, however large Nc_2 and Cy_2 may become, they increase in the same proportion to one another as Nc_1 and Cy_1, so that $\frac{Nc_2}{Cy_2}$, from the beginning of growth due to the second cycle, remains equal to $\frac{Nc_1}{Cy_1}$ and therefore to $\frac{Nc_1 + Nc_2}{Cy_1 + Cy_2}$.

From this it obviously follows that growth due to the second cycle cannot be initiated until the nucleo-cytoplasmic ratio in the cells which participate in the first cycle has fallen to the initial value characteristic of the cells of the second group, that is, the ratios which they possessed when they arose by division. They must remain in a condition of stasis until cells of a higher ratio have fallen to their level, when all can proceed together. Instances of such stasis in tissues or organs for long periods of development, followed by vigorous growth when the development of other tissues has reached the necessary stage are too numerous to require exemplification here. It will be admitted that the above picture of the course of growth and

[16] In the very earliest stages of embryonic development this, of course, will not be strictly true, but with these we are not here concerned. Our data reveal nothing regarding the time and weight relationships in very early embryonic development.

[17] It will, of course, be realized that however small Nc_2 and Cy_2 or Nc_3 and Cy_3 may individually be, their *ratios* may have any magnitude.

development corresponds in this particular very closely with the facts.

From these considerations, therefore, three conclusions emerge, namely:—

1. The cells which participate in the growth composing any autocatalytic growth-cycle have initially lower nucleo-cytoplasmic ratios than the cells which participate in the preceding cycles.

2. Cells of large nucleo-cytoplasmic ratios inhibit growth of cells which possess smaller ratios.[18]

3. Subsequently to the production of any cell by division, the increment of cytoplasm always stands in a fixed proportion to the *increment* of nucleus and this proportion is the same for all of the cells of which the organism is composed.

These conclusions offer a very welcome explanation, hitherto lacking, of the relative positions in time of the several autocatalytic cycles which contribute to the total growth of an animal.

In equations (1) and (2) it is easy to comprehend why A, the maximum growth attainable in any cycle, may differ in different cycles. Its magnitude is determined on the one hand by the abundance of available nutrients (the substrates of growth) and, on the other, by the equivalence of these substrates in terms of the products of the growth-process which constitutes that cycle. For since A is measured in terms of the *products* of the growth-process, being their final mass, if the products be such as are expensive to produce, either on account of the high velocity of the reverse reaction (breakdown of tissue elements when produced) or because of the large number of molecules of substrate required to participate in their formation,[19] then A for that

[18] Possibly by prior appropriation of nutrients, due to their higher metabolic rate (Robertson[2]).

[19] This would be the case, for example, in the synthesis of connective-tissue proteins which are of abnormal amino-acid composition, so that many molecules of protein of average composition might have to be decomposed into their constituent amino-acids to produce those required for the manufacture of a single molecule of the aberrant protein.

cycle will be small in comparison with its value in a cycle in which the products are less expensive to manufacture.[20]

Similarly, we can understand why k should vary in different cycles, because the reactants in each cycle differ from those in any other. But it is not immediately obvious what physico-chemical mechanisms determine the magnitudes of t_1, t_2 and t_3, the moments, namely, at which the autocatalytic growth-cycles attain their maximum velocities. These are clearly related to the moments at which the growth due to each cycle becomes of appreciable magnitude. Given the values of k, A and $t_{1,\ 2\ \text{or}\ 3}$ for any cycle, it is possible to compute the time (that is, age) at which the weight-increments due to that cycle must become of measurable magnitude. The value of t_1, however, is simply computed, in practice, from the observations. Its value has not hitherto been predictable from any other consideration, nor has it been possible to refer it to any determinative factors. The hypothesis outlined above establishes such factors and enables us to understand why the onset of later cycles is necessarily delayed until the preceding cycles have attained a certain stage of development.

The Relationship of Growth-Cycles to Differentiation.

The conclusions reached above, and derived from a comparison of the time-relations of growth with the corresponding changes of nucleo-cytoplasmic ratio, fit with remarkable precision into the hypothesis of differentiation which the author has put forward elsewhere, basing it upon data of totally different origin and description.[21]

It is a well-known fact that the early cleavage-cells produced at the beginning of the development of an embryo, are toti-potent, that is, they are individually capable, even in the absence of the other cleavage-cells, of giving rise to the complete embryo. At a comparatively early stage of development, however, usually within a few cell-divisions, this toti-potency is lost and becomes replaced by a partial capacity for

[20] The proportion of water incorporated within the living tissue would also constitute an important factor modifying the value of A. Attention has been drawn to this fact in comunications by Cramer, W., and Pringle, H., *Proc. Roy. Soc. London, Series B.*, 1910, lxxxii, 307, 315.

[21] Robertson.[2]

reproduction capable of replacing large portions of the embryo which may have been lost through mutilation. Progressively the reproductive potency of the cells of the embryo diminishes until, in the adult, it remains only to the extent that tissues possess the power of repairing injury to themselves.

The most natural interpretation of these facts, in the light of the chromosomal theory of inheritance, is that the chromosomal heritage of cells produced in later stages of development is incomplete, so that they retain only a corresponding fraction of the original power, possessed by the fertilised ovum, of reproducing the whole organism. If, however, the chromosomal endowment of the more recent cells is less than that which the parent cells initially possessed, this can only be because division occurred before the nuclear substance of the parent-cell had been completely reduplicated.[22] According to this hypothesis, therefore, differentiation, in the development of an organism, is attributable to the achievement by certain cells of the power of undergoing division at a lower nucleo-cytoplasmic ratio than that at which the parent-cells divided. Such cells must possess a different physiological character from their parents and react, in consequence, in a different manner to their environment. The reaction to the environment determines the type (*i.e.* structure and composition) of cytoplasm which is produced, so that from the altering nucleo-cytoplasmic ratios of successive generations of cells spring altering physiological responses and consequent differentiations of structure.

Obviously, if this were the case we would expect to find, at the conclusion of cell-production in development, various groups of cells which differ in their nucleo-cytoplasmic ratios, those of latest production being possessed of the lower ratios. The conclusions arrived at in this paper, and based upon quite independent evidence, also point to the existence, within the developing animal, of groups of cells which are distinguishable from one another by the possession of different nucleo-

[22] It must be recollected that the element of the nucleus which stains with hematoxylin is nucleic acid (Mathew, A. P., *Am. J. Physiol.*, 1898, i, 445). The chromosomes to which the geneticist traces the inheritable characters of the adult are therefore masses of nucleic acid, whatever else may chance to be associated with them.

cytoplasmic ratios. Our conclusions drawn from the study of growth itself and chemical estimations of the nucleo-cytoplasmic ratio contribute, therefore, very materially, to support the above theory of differentiation.

The Relation of the Nucleus to the Growth of Cytoplasm.

Confining our attention to a single asymmetrical cycle of growth, such as the first growth-cycle in the mouse, since we have found that the velocity constant of growth is given by the proportion:—

$$\frac{k^1}{k} = \frac{x + b}{x}$$

and we have also found that $\frac{k^1}{k}$ is proportional to $\frac{Nc}{Cy}$, that is, that $\frac{k^1}{k} = \rho \frac{Nc}{Cy}$ where ρ is a constant proportionality-factor, it follows that:—

$$\frac{x + b}{x} = \rho \frac{Nc}{Cy}$$

Now x, the weight of an animal is almost entirely[23] the weight of its cytoplasmic constituents and products. We may, therefore, with very close approximation to the truth, write Cy for x in the above equation, from which it follows that:—

$$Cy + b = \rho\, Nc$$

and hence that:—

$$\rho \frac{dNc}{dt} = \frac{dCy}{dt}$$

or, in other phraseology, that the growth of cytoplasm, subsequent to the production of the cell by the cell-division from which it arose, stands always in a fixed proportion to the growth of the nucleus, a conclusion to which we have already been impelled for other reasons.

[23] That is, over 90 per cent. In thymus, which represents a comparatively undifferentiated and richly nucleated type of tissue, the total nuclear constituents, chromosomal and others, constitute 15 per cent of the weight of the gland.

It also follows that, substituting $Cy + b$ for $x + b$ in equation (4):—

$$\frac{dx}{dt} = k\,\rho\,Nc(A - x)$$

in which k and ρ are constants and A is the maximum growth attainable in the cycle. From this we derive the very important and suggestive conclusion *that the growth of cytoplasm is determined by a monomolecular process which is catalysed by the nucleus in proportion to its mass*. The autocatalytic character of the growth-process in animals and plants takes its origin, therefore, in the fundamental dependence of cellular syntheses upon the presence of the nucleus or its products.

The error involved in assuming the identity of x (total growth achieved) with Cy (the mass of cytoplasmic material) is very small and diminishes, of course, as the nucleo-cytoplasmic ratio decreases.[24] The simple and fundamental character of the relationship deduced by making this approximation suggests, however, that this represents the true relationship and that the empirical formula:—

$$\frac{dx}{dt} = k(x + b)\,(A - x)$$

represents, in fact, a mathematical approximation. We may, in consequence, expect to find, as we approach the beginnings of embryonic development (when $\frac{Nc}{Cy}$ is at its maximum value and the error involved in neglecting Nc in comparison with Cy is therefore at its maximum also) that the autocatalytic equations, as hitherto formulated, no longer apply. The necessary measurements of very early embryonic

[24] If the chromosomal constituents of the nuclei were to represent as much as 10 per cent of the total weight of the organism, the departure from proportionality of nuclear to cytoplasmic increment would only be 1 per cent, for in equalising $\frac{Nc}{Cy}$ to $\frac{Cy+b}{Cy}$ instead of to $\frac{Cy+Nc+b}{Cy+Nc}$ only the *square* of the nuclear mass need be neglected to ensure proportionality of the nuclear and cytoplasmic increments. The actual error, during the period of development covered by post-natal growth-curves, is probably much less than this and therefore far within the magnitude of the experimental error of weight determinations in animals.

growth have not yet been carried out, however, with sufficient frequency and exactitude to permit the demonstration of these expected deviations from the autocatalytic formulæ.

The tendency of all of these results, therefore, is to indicate that the nucleus plays a predominant rôle in determining the growth of cytoplasm. This conclusion emerges, be it noted, solely from the quantitative analysis of the growth-process itself, coupled with the empirical correspondence between growth-velocity and nucleo-cytoplasmic ratios. This being the case it is a most noteworthy fact that the conclusions thus derived stand in such remarkable accord with the indications of modern genetic research. From the study of the time-relations of growth and the concurrent alterations of nucleic acid content of the animal, on the one hand, and from the study of cytology and inheritance upon the other, we attain to the same conception of the nucleus as the predominating factor in shaping the development of the cell in which it resides.

CONCLUSIONS.

1. It has previously been shown by the author and many others that growth, in animals and plants, is an autocatalysed process. In animals it is usual to find that growth occurs in several superimposed autocatalytic cycles. In many cases, in plants and animals, especially if the cycle is one which occupies a large proportion of the growing period, it is found that the velocity-constant of the autocatalysed monomolecular formula falls off as growth proceeds, at first rapidly and later more slowly.

2. It has previously been shown by the author that the fall of the velocity-constant of growth, in the white mouse, is directly proportional to the fall of the nucleo-cytoplasmic ratio, determined by the chemical method of Le Breton and Schaeffer. If we assume this relationship to be generally applicable to the growth of animals and plants, then the following additional conclusions may be deduced, without calling in the aid of any other assumption:—

3. The increase of cytoplasm in any given cycle of growth is proportional to the concurrent increase of nuclear material.

4. The growth of cytoplasm takes place in accordance with a monomolecular formula in which the velocity-constant varies directly as the mass of the nucleus.

If we superadd to these facts and deductions the hypothesis that each growth-cycle represents the growth of a separate group of cells within the animal, then the additional conclusions follow:—

5. That the cells which participate in the growth composing any cycle have initially lower nucleo-cytoplasmic ratios than the cells which participated in the preceding cycles.

6. That cells of large nucleo-cytoplasmic ratios in a multicellular animal inhibit the growth of cells which possess smaller ratios.

7. These conclusions collectively imply that the nucleus plays a predominant rôle in determining the development of the cell in which it resides.

THE THERMIC EFFECT OF DEATH.

By W. W. LEPESCHKIN.

(From the Department of Botany, University of Illinois, Urbana.)

(Accepted for publication, July 5, 1928.)

The principal chemical compounds which compose living matter (aside from water) presumably consist largely of protein substances and lipoids and are very unstable. As explosive substances they can be destroyed not only by chemical and physical but even by purely mechanical agents. A complete decomposition of these compounds brings about death.[1] As the decomposition of very unstable chemical compounds is usually accompanied by the production of heat, we may expect the decomposition of the principal compounds of living matter and death to be accompanied by the production of heat.

In this paper I intend to describe the results of my experiments on the thermic effect of death.

Material and Method.

In my experiments I used yeast which was mixed with distilled water. As it had only little oxygen and no sugar at its disposal its respiratory processes were inhibited and no production of heat could be observed if the mixture of yeast and water was placed in the calorimeter. The yeast used was Fleischmann's compressed yeast washed with distilled water. The calorimeter used was a Dewar flask closed by a rubber stopper through one of two openings of which a Beckmann thermometer was inserted. The second opening was made for the introduction of poisons into the flask. The flask was placed into a wooden box filled with shavings.

The Dewar flask was first used as a calorimeter by *Rubner*[2] who determined the heat produced by alcoholic fermentation and by the inversion of sugar. The volume of the flask was somewhat greater than that used by Rubner but the

[1] Lepeschkin, W. W., *Biol. Zentr.*, 1926, xlvi, 487; Kolloidchemie des Protoplasmas, Berlin, 1924, 155; *Arch. exp. Zellforsch.*, 1927, iv, 212; *Protoplasma*, 1927, ii, 239.

[2] Rubner, M., *Arch. Hyg.*, 1904, xlviii, 274; xlix, 335, 398.

walls were the same (the volume was 450 cc.). In the experiments described by Rubner, heat was produced very slowly, and the loss of heat by the flask, during the experiment, had to be taken into account. In my experiments the production of heat by death continued, however, for a very short time, not more than a few minutes. The loss of heat from the flask during the period of the experiment was therefore so inconsiderable that it could be disregarded.

I should like to cite here the results of my experiments concerning the constancy of temperature in the Dewar flask filled with water.

The position of mercury in the Beckmann thermometer was such that the temperature 2.300° corresponded to 20.9°C. The changes of temperature in the flask are given in the following table.

Date	Time	Temperature in the flask (Beckmann)	Temperature in the room (Centigrade)
October 13	12:30 p.m.	2.300	20.9
	3:00 p.m.	2.302	22.2
	4:00 p.m.	2.310	21.1
October 14	7:15 a.m.	2.290	20.3
	8:45 a.m.	2.293	22.8
	11:15 a.m.	2.303	22.6
	12:45 p.m.	2.321	22.8

From the cited results we may conclude that the change of temperature in the room produces no considerable effect upon the temperature in the flask. The greatest change was 0.031°C. and it occurred only after an interval of 5½ hours with the room temperature increased from 20.3° to 22.8°C. Since the temperature in the flask, during the experiment, did not exceed the room temperature more than 0.5°C. we may assume that it remained constant during the experiment which lasted only 5 or 10 minutes.

On the other hand in order to kill yeast cells I had to mix the poison with the yeast suspension, by stirring the liquid in the flask. It was therefore necessary to know whether the stirring changes the temperature of water in the flask. This stirring was produced, in my experiments, by an energic shaking of the flask.

Temperature in the flask (Beckmann)	Temperature after the first stirring	Temperature after the second stirring	Temperature after the third stirring
2.362	2.372	2.377	2.378
1.992	2.015	2.022	2.022
1.723	1.755	1.762	1.763
2.015	2.011	2.010	2.010

From the above experiment it is apparent that before the introducing of poisons the liquid must be shaken until the temperature of the liquid ceases to

change. Then we can be certain that the temperature will not be altered by mixing poisons with the liquid in the flask.

The death of yeast was produced, in my experiments, by adding either, corrosive sublimate or chloroform. In order to make the temperature of the poisons exactly equal to the temperature of the liquid in the flask the poisons were introduced into the flask in a special glass tube which remained there for about 1 hour before mixing the poisons with the liquid. This time is sufficient for an equalization of temperature of the poison and the liquid in the flask. The tube used (about 1½ cm. in diameter, 7 cm. long, volume 6½ cc.) was drawn out at the lower end to the diameter of 2 mm. and bent upwards (Fig. 1). The upper end of the tube was provided with a long rubber tube closed by means of a pinch-cock and inserted through the second opening of the rubber stopper of the flask. The tube was filled with a saturated solution of corrosive sublimate or with chloroform of the same temperature as the suspension in the flask. The volume of each was exactly 5½ cc. The poisons were introduced into the liquid by blowing into the rubber tube after opening the pinch-cock. A special experiment showed that such blowing did not alter the temperature in the flask.

FIG. 1

The action of sublimate on protoplasm has peculiarities which must be considered in the determination of the thermic effect of death. Sublimate alters living matter chemically producing salt like compounds with proteins. It is very probable that the formation of such compounds is accompanied by the production of heat, and this heat must be subtracted from the heat produced by the action of corrosive sublimate on yeast. The heat of the formation of compounds between sublimate and the proteins of yeast could be determined on the following basis.

Mercury bichloride acts on proteins of all kinds in the same manner. Hydrogen of the carboxyl groups of proteins is replaced by mercury, so that a salt-like compound arises in all cases. On the other hand, as is known, the formation heats of salts of different organic acids which possess an analogous structure (with the same metal) are approximately equal.[3] The formation heats of salt-like compounds of mercury with different proteins are therefore alike. Thus in order to determine the heat produced in the reaction between sublimate and the proteins of yeast we can limit ourselves to a determination of

[3] Berthelot, M., *Ann. chim. et phys.*, 1875, vi, series 5, 326. Massol, G. 1894, i, series 7, 145.

heat of the reaction between sublimate and any common protein, as for instance, albumin. Both heats must be approximately equal.

The Thermic Effect Produced in the Reaction between Corrosive Sublimate and Albumin of Eggs.

40 gm. albumin of eggs (Merck) were dissolved in 400 gm. distilled water, and the solution (volume 425 cc.) was poured into the Dewar flask. Then the glass tube (Fig. 1) filled with a saturated solution of sublimate was introduced into the flask, and the latter was closed with the rubber stopper. In 1 hour the temperature in the flask was 1.723. After an energic stirring the temperature increased to 1.750; after the second stirring it was 1.762 and after the third stirring it was 1.767; further stirring did not alter this temperature. Immediately the sublimate solution was blown out of the tube into the albumin solution, and the mixture was stirred. The temperature increased at once to 1.796. The further stirring raised the temperature to 1.820, but it at once began to decrease. Therefore we can conclude that the heat produced in the reaction between sublimate and albumin warmed the liquid in the flask 0.053°C. At the same time it warmed the thermometer and the flask itself.

The specific heat (heat capacity) of the albumin solution (10 per cent) is about 0.9; as to 425 cc. of this solution 5.5 cc. solution of mercury chloride was added and as the specific heat of diluted solutions of mercury chloride is almost equal to that of water the heat which is necessary to warm the liquid in the flask 0.053°C. is $430 \times 0.9 \times 0.053 = 20.5$ gm. calories. The Beckmann thermometer contained about 40 gm. mercury, and it therefore absorbed about $40 \times 0.03 \times 0.053 = 0.06$ gm. calories. Concerning the heat absorbed by the flask my experiments showed, in accordance with Rubner[2] (page 372), that it is about $15 \times 0.053 = 0.79$ gm. calories. The total heat produced in the reaction was about 21 gm. calories or per gram dry albumin $21 \div 40 = 0.52$ gm. calories.

We shall now describe the results of the determination of the heat produced by the death of yeast.

The Thermic Effect of Death Produced by Corrosive Sublimate.

82.44 gm. yeast (washed and freed from water by pressure) were mixed with 400 gm. distilled water. The mixture was stirred until all yeast cells were separated from each other. Then it was poured into the Dewar flask. The yeast contained 64 per cent water and, therefore the dry substance of the yeast used was 29.68 gm. After stirring the liquid several times the final temperature was 4.014°, that is 22.6°C. Soon after the glass tube (Fig. 1) filled with a satu-

rated solution of sublimate of the same temperature was introduced into the flask and the flask was closed by the rubber stopper.

In 1 hour the liquid in the flask was stirred again several times, and the final temperature was 4.017. Immediately the solution of mercury chloride is blown out from the glass tube into the suspension of yeast, and the liquid again stirred. The temperature of the liquid increased at once to 4.090, the maximal temperature.

The microscopical investigation of the yeast taken from the flask showed that only 50 per cent of the cells were killed by sublimate (test by methylene blue). The size of the Dewar flask did not permit, however, the use of a greater quantity of mercury chloride. The tube used contained only 5.5 cc. of solution.

From the cited results we can conclude that the heat produced by the action of sublimate on yeast warmed the liquid in the flask, the thermometer and the flask itself 0.073°C. According to Rubner[3] (page 372) the specific heat of yeast is 0.785. Therefore the heat produced by the action of corrosive sublimate on the yeast is: $0.073 (405 + 82.4 \times 0.785 + 40 \times 0.03 + 15) = 35.4$ gm. calories or per gram of dry yeast $35.4 \div 29.68 = 1.19$ gm. calories. Subtracting from this value the heat produced in the reaction between mercury chloride and protein substances of the yeast (dry yeast contains about 50 per cent protein substances[4] and only 50 per cent of the cells were killed) that is $0.52 \times 0.5 \times 0.5 = 0.13$ gm. calories we reach the conclusion that there was an additional effect amounting to $1.19 - 0.13 = 1.07$ gm. calories per gram of dry yeast, or about 2 gm. calories per gram of the dry substance of killed yeast (50 per cent of the cells only were killed).

The Thermic Effect of Death Produced by Chloroform.

As is known chloroform does not form chemical compounds with protein substances, but it is taken up by protoplasm and strongly accelerates the denaturation of protein substances. Such denaturation may bring about death.

80 gm. yeast which contained 24.8 dry substance were mixed with 400 gm. distilled water, and the mixture was stirred until all cells

[4] König, J., Chemie der menschlichen Nahrungs- und Genussmittel, Berlin, 1919–23, 1056.

were separated from each other. Then the mixture was poured into the Dewar flask. The temperature in the flask was, after stirring several times, 0.825° (Beckmann). The glass tube (Fig. 1) containing 5 gm. chloroform was then introduced into the flask, but the chloroform was not blown out of the tube.

In 1 hour the liquid in the flask was stirred again several times, and the final temperature was 0.875°. Immediately chloroform was blown out from the glass tube into the suspension of yeast, and the mixture again stirred. The temperature rose at once to 0.910, and this temperature was not altered by the further stirring of the mixture. The temperature rise is therefore 0.035°C.

We can therefore conclude that the death of yeast was accompanied by the production of heat of $0.035\ (400 + 5.5 \times 0.23 + 80 \times 0.785 + 1.2 + 15) = 16.7$ gm. calories, or per gram of killed yeast $16.7 \div 24.8 = 0.67$ gm. calories. As the microscopical investigation showed that only 50 per cent of the yeast cells were killed this heat per gram of killed yeast is about 1.5 gm. calories. The obtained value is somewhat smaller than that obtained in the former experiment, but the difference does not exceed the experimental error.

The Action of Corrosive Sublimate and Chloroform on Dead Yeast.

Up till now we considered the thermic effect produced by the action of mercury bichloride and chloroform on living yeast as a consequence of death, that is, of the decomposition of the principal chemical compounds composing living matter. In the case of bichloride this effect is, at the same time, a consequence of the reaction between bichloride and proteins of yeast; but the latter thermic effect proved to be small in comparison with the effect produced by death.

One could suppose however that the thermic effect which we ascribe to death could perhaps be produced by other unknown processes in which both poisons possibly play a part, as for instance, by an adsorption of them by the substances of yeast and so on. I should like therefore to show here that the thermic effect observed is produced by death itself. Indeed, if the same manipulations are repeated on dead yeast we either observe no thermic effect or this effect corresponds only to the reaction between mercury bichloride and proteins of yeast.

80 gm. yeast (washed and freed from water by pressure) were mixed with 400 gm. distilled water. The mixture was stirred until all yeast cells were separated from each other. Then it was heated to 80°C. so that all yeast cells were killed. After the suspension took the temperature of the experimental room it was poured into the Dewar flask. The yeast contained 25.5 gm. dry substance.

After stirring the liquid several times the final temperature was 2.395° (Beckmann). Soon after the glass tube (Fig. 1) filled with a saturated solution of corrosive sublimate of the same temperature was introduced into the flask and the flask was closed by the rubber stopper.

In 1 hour the liquid in the flask was stirred again several times, and the final temperature was 2.417°. Immediately the solution of sublimate (5.5 cc.) was blown out of the glass tube into the suspension of dead yeast, and the liquid again stirred. The temperature of the liquid increased at once to 2.423°. The further stirring did not alter this temperature.

From the cited results we can conclude that the heat produced by the action of sublimate on dead yeast warmed the liquid in the flask, the thermometer, and the flask itself 0.006°C. Therefore the heat produced was $0.006\ (400 + 82.4 \times 0.785 + 20 \times 0.3 + 15) = 3$ gm. calories or per gram of dry yeast $3 \div 25.5 = 0.12$ gm. calories. This heat corresponds to the heat produced in the reaction between albumin and mercury bichloride (0.13 gm. calories).

No effect of chloroform on dead yeast could be observed. The experiment was made in the same manner as that with mercury bichloride. The final temperature in the Dewar flask containing a tube filled with 5.5 cc. chloroform was 2.479°. After mixing chloroform with the suspension of yeast and stirring several times it did not change at all.

The Thermic Effect of Death as Compared with Heat Produced in Other Chemical Reactions.

The thermic effect of death observed in my experiments is, of course, very small as compared with heat produced in most of the

chemical exothermic reactions. The heat produced by the decomposition of HI to H and I is accompanied, for instance, by the production of 46 gm. calories per gram, and the decomposition of N_6Hg_3 gives 290 gm. calories per gram.

If we consider however the molecular weight of the principal compounds of living matter as very great, we come to the conclusion that the heat produced by the decomposition of 1 molecule of these compounds is of the value which could be compared only with values observed on explosives. Indeed the principal compounds of living matter contain proteins as a component part, but the molecular weight of the latter is very great, as for instance, the molecular weight of hemoglobin (determined in the chemical analysis according to the content of iron) is 16,669. The molecular weight of the principal chemical compounds of living matter is certainly still greater. Thus if the molecular weight of these compounds had been even like that of hemoglobin the heat produced by the decomposition of 1 gm. molecule of these compounds would at least be $3 \times 16{,}669$,* that is greater than 40 kilo calories, while the heat produced by the destruction of 1 gm. molecule of HI is only 6 kilo calories, that of NCl_3 is 38 kilo calories, and so on. Therefore, on the average, the heat produced by the decomposition of 1 gm. molecule of the principal compounds of living matter is more than that of such an unstable and explosive body as NCl_3. Thus it is very comprehensible that the molecule of the principal chemical compounds composing living matter can be destroyed not only by chemical and physical but also by purely mechanical agents. This molecule is so unstable as to be compared with the molecules of explosive substances.

SUMMARY.

The thermic effect of death as observed on yeast is about 2 gm. calories per gram of dry substance and is therefore very small as

* The thermic effect of death is, on the average, 1.7 gm. calories per 1 gram of dry yeast. If we suppose that even all protein substances (50 per cent) and lipoids (5 per cent) form the principal compounds (aside from water) of its living matter we must conclude that the thermic effect of death is 1.7:0.55 = 3.1 gm. calories per gram of these compounds.

compared with the heat produced in most of the exotherm chemical reactions. As the chemical compounds composing living matter have a very great molecular weight, the decomposition of their molecule which brings about death produces more heat than the decomposition of the molecule of some explosive substances. This explains the great instability of the principal compounds of living matter.

Addendum.—After the correction of the proof my attention was drawn to two papers concerning the problem discussed above. In one of these papers Peters describes his experiment in which muscles were poisoned with chloroform.[5] The heat produced hereby (calculated per gram of dry substance) is approximately equal to the heat observed in my experiments on yeast. Muscles can be considered as consisting of living matter, and therefore Peters' results are in complete accord with my results. In the other paper Meyerhof tried to determine the heat produced by the action of acroleine on erythrocytes;[6] the poisoning was extended until the respiration irreversibly ceased. Meyerhof admitted that he observed the death of erythrocytes, though, according to his description, no hemolysis was observed; indeed Meyerhof writes: "Wird dasselbe in genügend hoher Konzentration zu lebenden Zellen zugesetzt, so tritt eine totale Atmungshemmung ein, die bei einem wenige Minuten später stattfindenden Wegwaschen des Giftes nicht mehr zurückgeht." A washing out of poison can certainly only be performed if no hemolysis occurs. It is possible that the heat produced by the death of erythrocytes is smaller than 1.4 gm. calories, as Meyerhof admits (1.4 calories is the limit of the exactness of his experiments), but it is more probable that the erythrocytes were not dead in Meyerhof's experiments, because the death of erythrocytes brought about by acroleine must be accompanied by hemolysis.

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[5] Peters, R. A., *J. Physiol.*, 1913–14, xlvii, 262.

[6] Meyerhof, O., *Arch. ges. Physiol.*, 1912, cxlvi, 181.

THE DEATH WAVE IN NITELLA.

II. Applications of Unlike Solutions.

By W. J. V. OSTERHOUT and E. S. HARRIS.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Accepted for publication, June 27, 1928.)

Previous experiments lead us to expect that protoplasm will give a negative current of injury when in contact with dilute solutions and that the opposite will be true with concentrated solutions. This should enable us to predict what will happen when a cell is brought simultaneously in contact with both kinds of solutions. It is shown in this paper that such prediction is possible.

These predictions involve the assumption that the protoplasm is composed of layers, *i.e.* an outer (*X*) and an inner (*Y*) both of which are probably non-aqueous and between these an aqueous layer (*W*). As previously explained[1] the more rapid alteration of the outer layer produces a positive current of injury and the more rapid alteration of the inner gives a negative current of injury. The latter is observed when the protoplasm is in contact externally with a solution less "effective"[2] than sap (*e.g.* 0.001 M KCl); the former occurs with solutions more effective than sap (*e.g.* 0.1 M KCl.) Hence if we apply 0.001 M KCl to the cell the outer layer (*X*) will be in contact with a less effective solution than the inner (*Y*) (which is in contact with sap).

If we arrange an experiment as in Fig. 1 with 0.001 M KCl at *A* and 0.1 M KCl at *C*,[3] and cut at *Z* we shall expect that, as previously described,[1] the death wave which starts at *Z* will pass rapidly down the cell to *C*. At *A*, where the protoplasm is in contact with 0.001 M KCl, we shall expect the inner layer (*Y*) to go first so that the protoplasm will at first become more negative[1] but at *C*, where it is in contact with 0.1 M

[1] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928–29, xii, 167.

[2] The more effective solution is the one which causes a more rapid alteration.

[3] The technique has been fully described in previous papers. Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 391, 417, 673; 1928–29, xii, 167.

KCl, the alteration of X will be more rapid than that of Y and in consequence the protoplasm will first become more positive.

That this is really so can be shown by arranging an experiment as in Fig. 2, killing B with 0.01 M KCl saturated with chloroform and leading off from A (in contact with 0.001 M KCl) to B and from C (in contact with 0.1 M KCl) to B: on cutting at Z we obtain approximately the "true" curves of A and C (*i.e.* the curves obtained when the value at B is zero), as shown in Fig. 3 *a*.[4] If we lead off from A to C we obtain

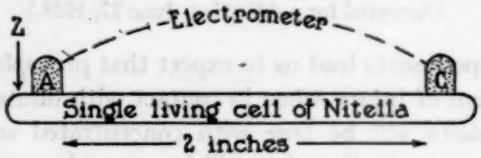

FIG. 1. Diagram to show the arrangement of certain experiments. At A and C are flowing contacts, or pieces of cotton soaked in solutions, or cups containing solution.

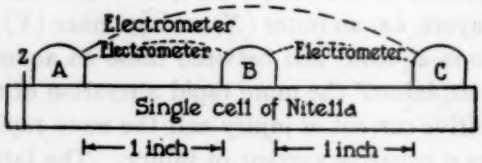

FIG. 2. As in Fig. 1 with an additional contact at B which is connected with A and with C.

approximately the difference between the "true" curves of A and C, as shown in Fig. 3 *b*.

The result corresponds to our prediction, *i.e.* at A (which is in con-

[4] The values at A will be more positive than they should be by a small but definite amount because of an effect due to the cell wall (see Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 673) which makes A appear more positive. This effect will remain constant until sap diffusing out at B raises the concentration of the solution imbibing the cell wall; the effect of this will be to make A appear still more positive but in very short experiments it may be neglected. What is said of A applies equally to C (in contact with 0.1 M KCl) except that the cell wall effect will make C appear somewhat more negative than it should be at the start and the coming out of sap at B will decrease this effect. These effects are small and may be neglected for our present purpose.

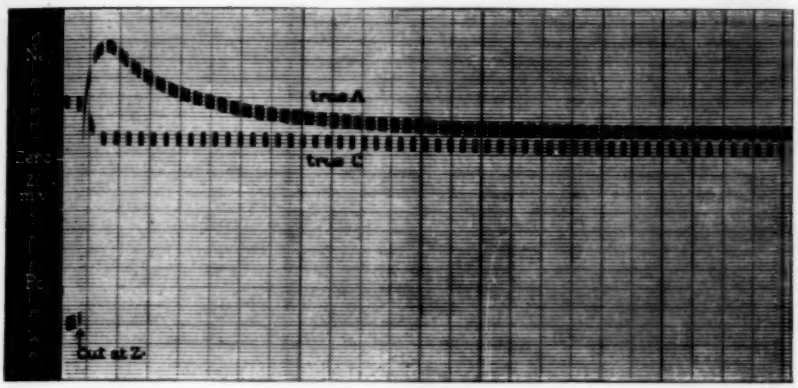

FIG. 3 *a*.

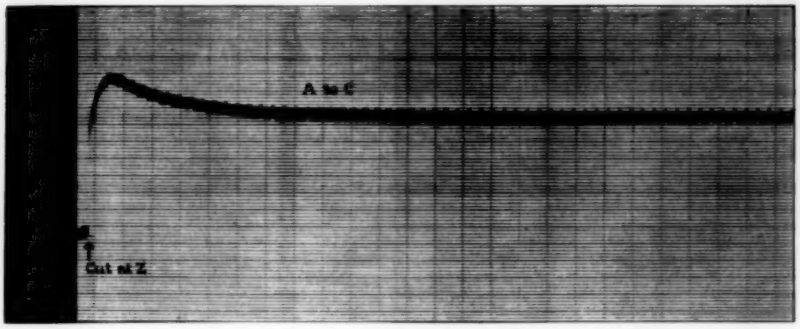

FIG. 3 *b*.

FIGS. 3 *a* and *b*. Photographic records showing potential differences, the experiment being arranged as in Fig. 2 with 0.001 M KCl at *A*, 0.01 M KCl at *B*, and 0.1 M KCl at *C*. The record starts after *B* has been killed (with 0.01 M KCl saturated with chloroform) so that *A* and *C* show their "true" values. On cutting at *Z* we see (in Fig. 3 *a*) that *A* becomes negative and then approaches zero while *C* becomes positive and then approaches zero. Curves *A* and *C* are recorded on one instrument while the *A* to *C* curve is simultaneously recorded on another (Fig. 3 *b*): this curve is approximately equal to the difference between the "true" curves of *A* and *C*.

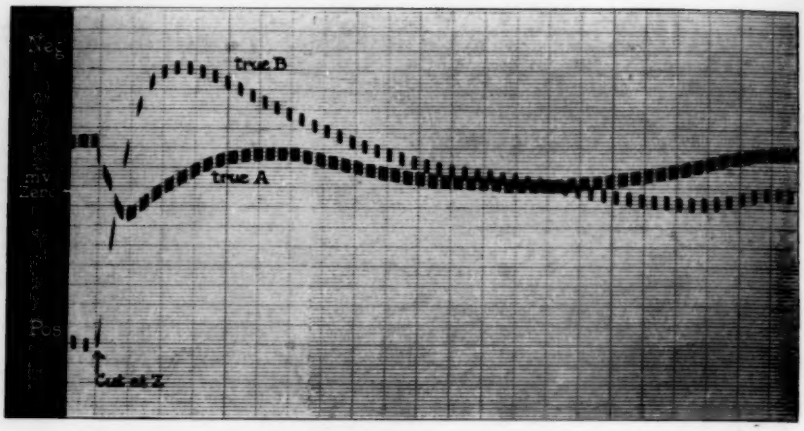

FIG. 4 *a*.

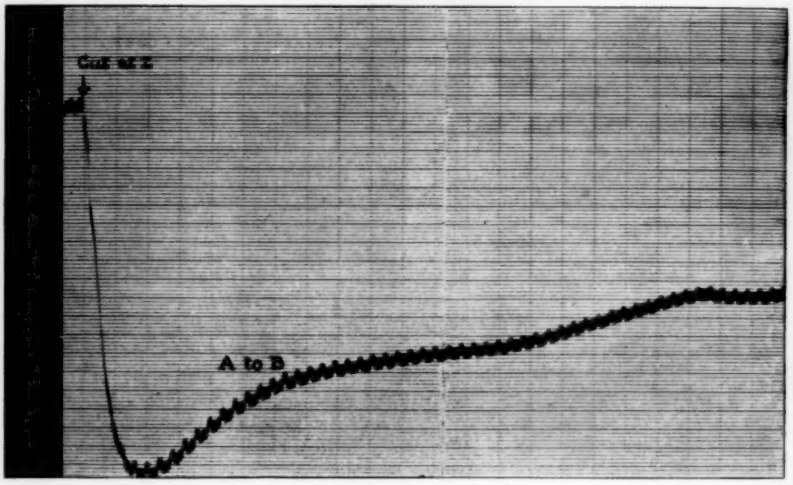

FIG. 4 *b*.

FIGS. 4 *a* and *b*. Photographic records showing potential differences, the experiment being arranged as in Fig. 2 with 0.1 M KCl at *A*, 0.001 M KCl at *B*, and 0.01 M KCl at *C*. The record starts after *C* has been killed (with 0.01 M KCl saturated with chloroform) so that *A* and *B* show their "true" values. On cutting at *Z* we see (in Fig. 4 *a*) that *A* becomes positive and then approaches zero while *B* becomes negative and then approaches zero. Curves *A* and *B* are recorded on one instrument while the *A* to *B* curve is simultaneously recorded on another (Fig. 4 *b*): this curve is approximately equal to the difference between the "true" curves of *A* and *B*.

tact with a solution less effective than sap) the protoplasm first becomes more negative as the result of the cut, indicating that the layer *Y* goes first; at *C* (which is in contact with a solution more effective than sap) the protoplasm first becomes more positive, indicating that the layer *X* goes first.

It is desirable to supplement this experiment by placing 0.1 M KCl at *A*, 0.001 M KCl at *B*, and then killing *C:* the result is shown in

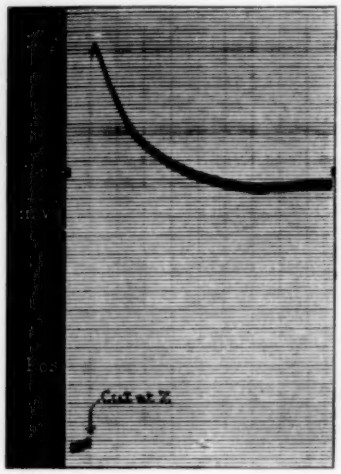

FIG. 5.

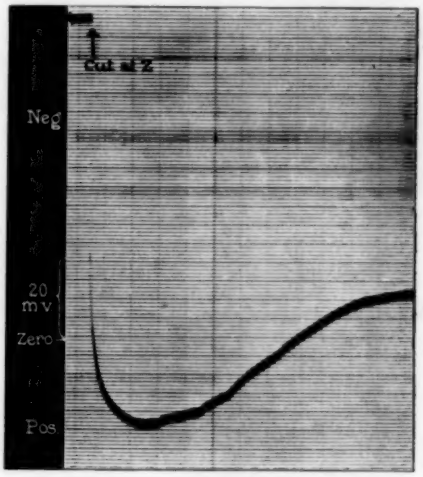

FIG. 6.

FIG. 5. Photographic record of potential differences, the experiment being arranged as in Fig. 1 with 0.001 M KCl at *A* and 0.1 M KCl at *C*. On cutting at *Z* we obtain a curve resembling that in Fig. 3 *b*.

FIG. 6. Photographic record of potential differences, the experiment being arranged as in Fig. 1 with 0.1 M KCl at *A* and 0.001 M KCl at *C*. On cutting at *Z* we obtain a curve similar to that in Fig. 4 *b*.

Figs. 4 *a* and 4 *b* (since *C* is killed we obtain the "true" curves of *A* and *B*). The result is again in accordance with prediction.

It may be thought that killing a spot may alter the condition of the cell and so affect the curves. We have accordingly performed experiments (arranged as in Fig. 1) in which the killing is omitted: these are illustrated by Figs. 5 and 6 which resemble Figs. *3 b* and 4 *b* sufficiently to indicate that the killing of a single spot makes no essential

difference. In all these cases we are dealing with the difference between two spots, A and C, or A and B, which are affected in turn by a death wave which starts from Z and travels down the cell.

An interesting experiment is shown in Fig. 8. The experiment was arranged as in Fig. 7 with 0.1 M KCl at A, 0.001 M KCl at B, and 0.05

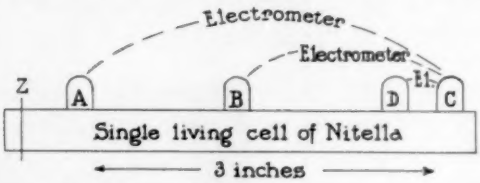

FIG. 7. As in Fig. 1 with additional contacts at B and D.

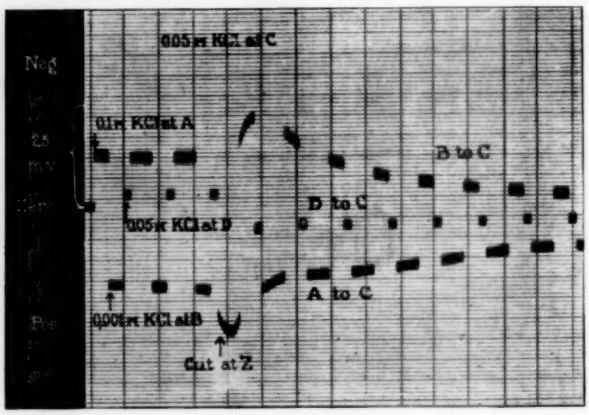

FIG. 8. Photographic record of potential differences, the experiment being arranged as in Fig. 7 with 0.1 M KCl at A, 0.001 M KCl at B, 0.05 M KCl at D and at C. On cutting at Z we see that A becomes positive and then approaches zero, B becomes negative and then approaches zero; D shows little change because it is in this case very near C and is in contact with the same solution: it becomes slightly positive (as expected) and then approaches zero.

M KCl at D and at C. At the start A is negative and B positive due to the concentration effect.[5] On cutting at Z, A changes from negative to positive and B from positive to negative. D shows little change

[5] This is discussed in Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 673.

because it is very close to *C* and is in contact with the same solution (its curve is in accordance with expectation[1]).

The fact that *B* becomes negative on cutting is an argument against the theory mentioned in a previous paper[6] that injury causes an instantaneous exit of sap which might account for the results without the necessity of assuming the presence of layers in the protoplasm. The coming out of sap at *B* could not in any case do more than bring *B* to the same condition as *C* and so reduce the potential difference between them to zero; it could not make *B* negative as is the case here (since sap in these experiments acts like 0.05 M KCl).

A great variety of combinations were tried which need not be described in detail since the results were in every case in accord with the theory previously developed. We may conclude that the theory serves a useful purpose in bringing all the facts under a single viewpoint and in enabling us to predict the behavior of the cell under a great variety of conditions.

SUMMARY.

The hypothesis of protoplasmic layers enables us to predict the bioelectrical behavior of the cell under a great variety of conditions. It is shown in the present paper that this is clearly the case when a death wave passes through different points in contact with unlike solutions.

[6] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 673.

THE VISCOSITY OF BLOOD SERUM, AS A FUNCTION OF TEMPERATURE.

By P. LECOMTE du NOÜY.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York, and the Pasteur Institute, Paris, France.)

(Accepted for publication, June 16, 1928.)

The viscosity of colloidal solutions is not easily determined by the methods which involve the use of a glass capillary. The technical difficulties encountered are important, and when they are successfully surmounted they still require a relatively large amount of liquid. Moreover, they do not permit the continuous observation of the variations which may take place in a solution as a consequence of a reaction, or under the influence of temperature, or of time. These were the main reasons which led us to establish a viscometer which would escape the above limitations. Such an instrument was described a few years ago.[1] Suffice it to say that it is based on the principle of two coaxial cylinders; the outside cylinder or cup, which contains about 1 cc. of the solution to be studied, is rotating at constant speed, and the inside cylinder, or bob, is suspended by means of a galvanometer wire. A mirror, supported by the suspension, a lamp and scale for the readings and a good thermostatic arrangement, are provided.

The purpose of the present paper is to report the results of roughly 167 series of experiments, representing about 17,000 readings of viscosity, performed with normal blood serum (rabbit, dog, horse), and to discuss the interpretation of some of the data obtained, with especial reference to the hydration of the serum proteins.

EXPERIMENTAL.

In order to save the reader's time, and to make him visualize a series of experiments at a glance, only charts will be published. The experiments chosen for publication are representative of the others, as no radical departure from the mean has been observed so far.

[1] du Noüy, P. L., *J. Gen. Physiol.*, 1923, v, 429.

The experiments were carried on in the following way: the serum was poured into the cup (1 cc.) and the bob lowered into the serum. The zero being checked, the motor was started, and a first reading made at the starting temperature (between 20° and 25°C.). After checking the zero again, and repeating this measurement at least 3 times, with an interval of 5 minutes between each reading, the heat was put on. (A current of 1 ampere through a resistance immersed in the oil surrounding the cup brought the temperature up from 20° to 70° in about 45 minutes.) Then the readings were taken, and recorded simultaneously with the temperature. A telescope placed alongside of the scale makes this possible. As a rule, unless some critical point was neared or some unforeseen phenomenon occurred, readings were taken every 2°. The current was left on until the temperature of 70°C. was reached, in the first series of experiments (Figs. 1 and 2); in all other experiments reported in this paper the heat was stopped after a certain temperature had been reached (50°, 55°, 56° to 60°) and the oil allowed to cool by itself. As a rule, in order that the cooling from, say, 55° to 20°, should take about the same time as the heating from 20° to 55°, cold water was made to circulate in the double wall of the oil bath, and the rate controlled accordingly.

The first set of experiments is shown in Fig. 1. The ordinates express the readings on the scale, and therefore are arbitrary figures, but proportional to the absolute viscosities. In order to give an idea of the order of magnitude, the curve expressing the viscosity of water as a function of temperature is drawn below.

A simple glance at this chart immediately reveals the presence of a critical point, corresponding to an absolute minimum of viscosity, at a temperature near 56°. Had we not known beforehand that this was a critical temperature for the serum, from a biological standpoint, we could not have failed to notice it from these curves.

Fig. 2 illustrates the same phenomenon. The turning point may vary, from 56° to 58°. But in the great majority of cases, the viscosity reaches its minimum value between 56° and 57°, stays constant up to 58°, sometimes up to 59°, and begins to increase more rapidly than it decreased before. From 62° to 65° the increase becomes very rapid, and it sometimes happens that the spot has left the scale before 70° is reached. This is usually the case with horse serum (which is normally more viscous than rabbit serum). It happens rarely in the case of rabbit serum. Fig. 2 shows that it was necessary to keep the temperature at 70° for 2 minutes in order to send the spot off the scale. It is to be noted that when fresh serum is used, important fluctuations

are frequently observed around 45° to 55°. These were *never* observed when the serum had been submitted to a heat of 55°, even for 5

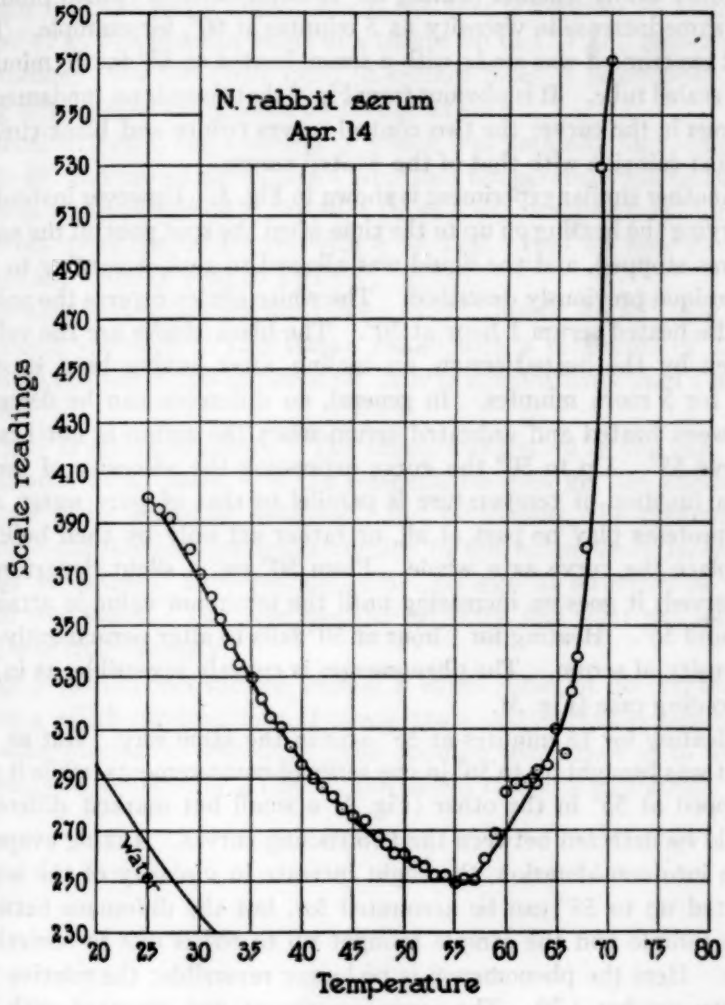

FIG. 1.

minutes, or when it was old. It appeared to us that it might be interesting to follow the phenomenon more closely, and to try and de-

termine whether the temperature played a specific part in the changes of viscosity, or whether the time of heating was the capital factor. In other words whether heating for 15 minutes at 55° would produce the same increase in viscosity as 5 minutes at 60°, for example. The first experiment was made with a serum heated at 55° for 10 minutes in a sealed tube. It is obvious from Fig. 2 that there is no fundamental change in the curve: the two control curves (white and black circles) almost coincide with that of the heated serum.

Another similar experiment is shown in Fig. 3. However instead of carrying the heating on up to the time when the spot goes off the scale, it was stopped, and the liquid was allowed to cool, according to the technique previously described. The white circles express the values of the heated serum 1 hour at 50°. The black circles are the values taken by the heated serum, on cooling, after having been kept at 57° for 5 more minutes. In general, no difference can be detected between heated and unheated serum when the serum is not heated above 55°. Up to 50° the curve expressing the viscosity of serum as a function of temperature is parallel to that of pure water, and its proteins play no part at all, or rather act only by their bulk, to displace the curve as a whole. From 50° on, a slight departure is observed; it goes on increasing until the minimum value is attained around 56°. Heating for 1 hour at 50° fails to alter permanently the viscosity of serum. The phenomenon is entirely reversible, as in the preceding case (Fig. 3).

Heating for 15 minutes at 55° acts in the same way. But as the heat was brought up to 56° in one series of measurements, while it was stopped at 55° in the other (Fig. 4) a small but marked difference could be detected between the two cooling curves. Taking evaporation into consideration, the slight increase in viscosity of the serum heated up to 55° can be accounted for, but the difference between this sample and the sample brought up to 56° is due to something else. Here the phenomenon is no longer reversible; the relative viscosity reaches 1.70. The same experiment was repeated with the same serum, heated for 15 minutes at 56° in a sealed tube. The mean viscosity was 1.70. The increase is small, but constant with this serum. It was not always observed with other sera. We can therefore state that, from our experiments, it appears that 56° is the lowest

temperature at which an irreversible phenomenon affecting its viscosity occurs in rabbit serum, in 15 minutes. However, an exception to this rule was found once. But if the heat is kept for 30 minutes at 56° (Fig. 5) the mean value of η climbs up to 1.77 and higher still on cooling. On the other hand in certain cases, 5 minutes at 56° (dog serum) may bring forth no change at all.

Another serum, the viscosity of which was normally high, heated up to 58° and cooled immediately after that temperature was reached, showed no modification. Serum 3 (η = 1.63) heated at 58° for 15 minutes, gave similar curves with η = 1.80. Half an hour at 58° brings the value of η up to 1.95.

Another serum heated for 1 hour, and 2 hours, at 58° (Fig. 6) reaches a viscosity of 1.85 (mean value) with a maximum of 1.90. 1 hour's heating of this serum at 60° fails to affect it more than 1 hour at 58°.

Fig. 7 is self-explanatory. The serum was heated in the cup, for 5 minutes, in all cases. As the temperature goes up, the different samples show a similar behavior. But on cooling, the differences between the different curves, according to the temperature reached, go on increasing. However, up to 62° no sign of structure in the liquid was found. The zero checked perfectly, and what was measured was true viscosity.

Fig. 8 summarizes for one animal a whole series of observations, showing which combinations of temperature and of time determined the same increase in viscosity. The question then arises as to what the increase in viscosity is due to, and how it can be interpreted.

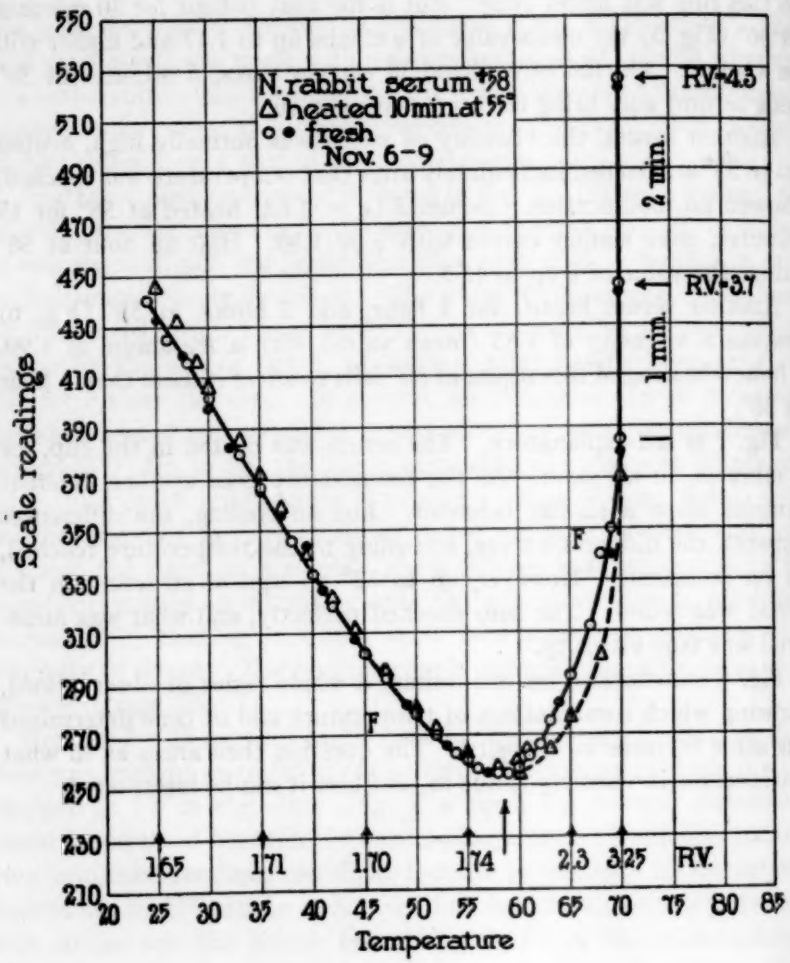
N. rabbit serum +58
△ heated 10 min. at 55°
○ ● fresh
Nov. 6–9
R.V.=4.3
R.V.=3.7
2 min.
1 min.
F
F
1.65
1.71
1.70
1.74
2.3
3.25
R.V.
Scale readings
Temperature
550
530
510
490
470
450
430
410
390
370
350
330
310
290
270
250
230
210
20 25 30 35 40 45 50 55 60 65 70 75 80 85

FIG. 2.

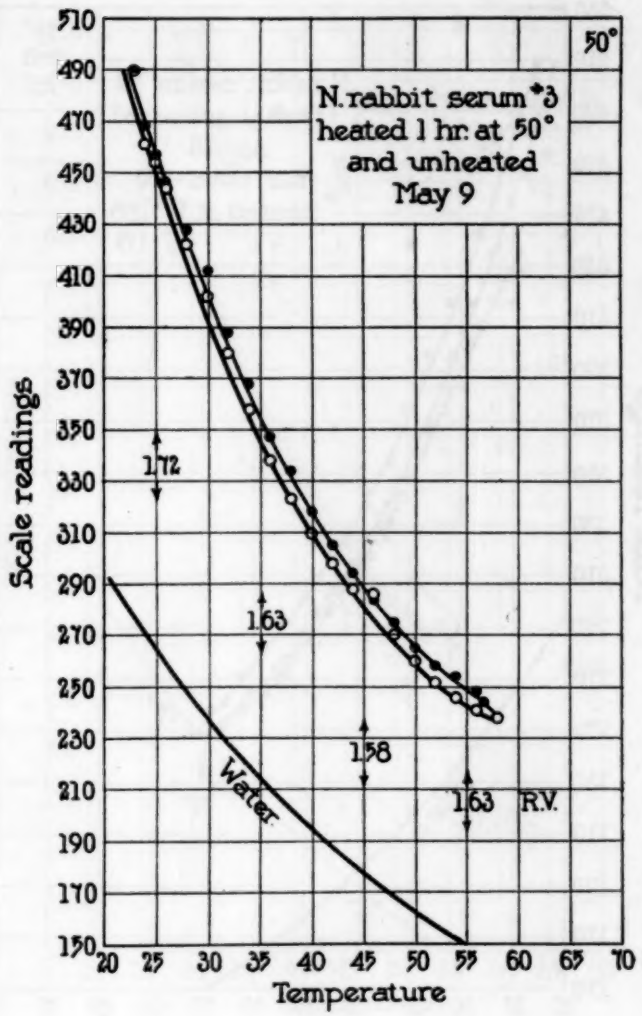
50°
N. rabbit serum *3
heated 1 hr. at 50°
and unheated
May 9
Scale readings
Temperature
1.72
1.63
1.58
1.63 R.V.
Water
510
490
470
450
430
410
390
370
350
330
310
290
270
250
230
210
190
170
150
20 25 30 35 40 45 50 55 60 65 70

FIG. 3.

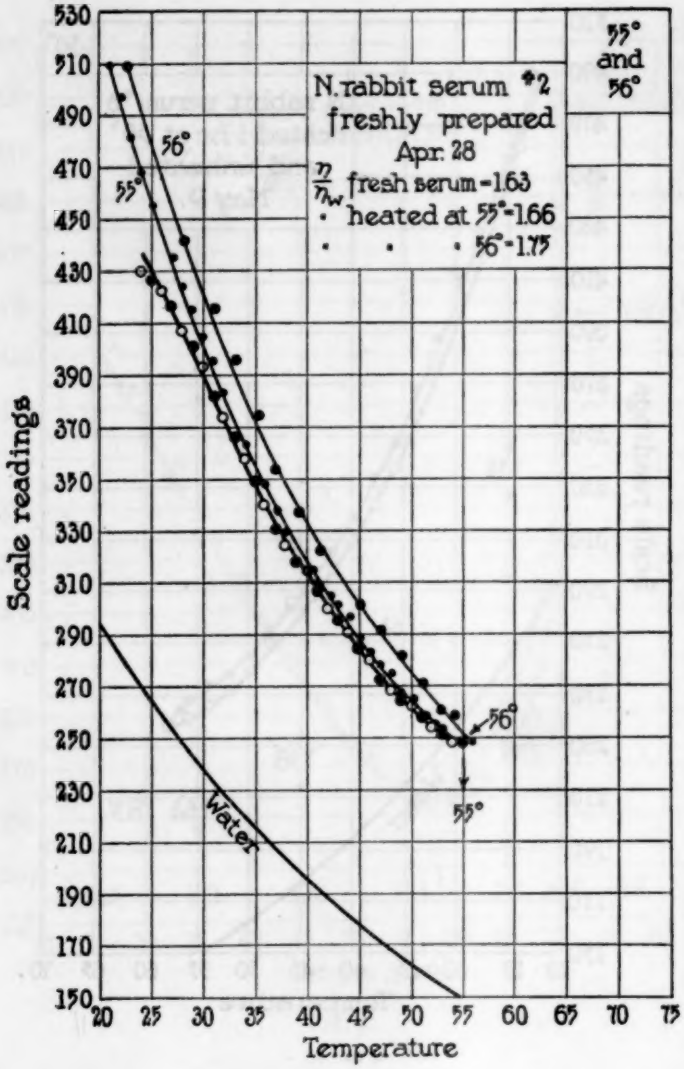
55° and 56°
N. rabbit serum #2
freshly prepared
Apr. 28
η/η_w fresh serum - 1.63
heated at 55° - 1.66
56° - 1.75
56°
55°
Water
Scale readings
Temperature
530
510
490
470
450
430
410
390
370
350
330
310
290
270
250
230
210
190
170
150
20 25 30 35 40 45 50 55 60 65 70 75

FIG. 4.

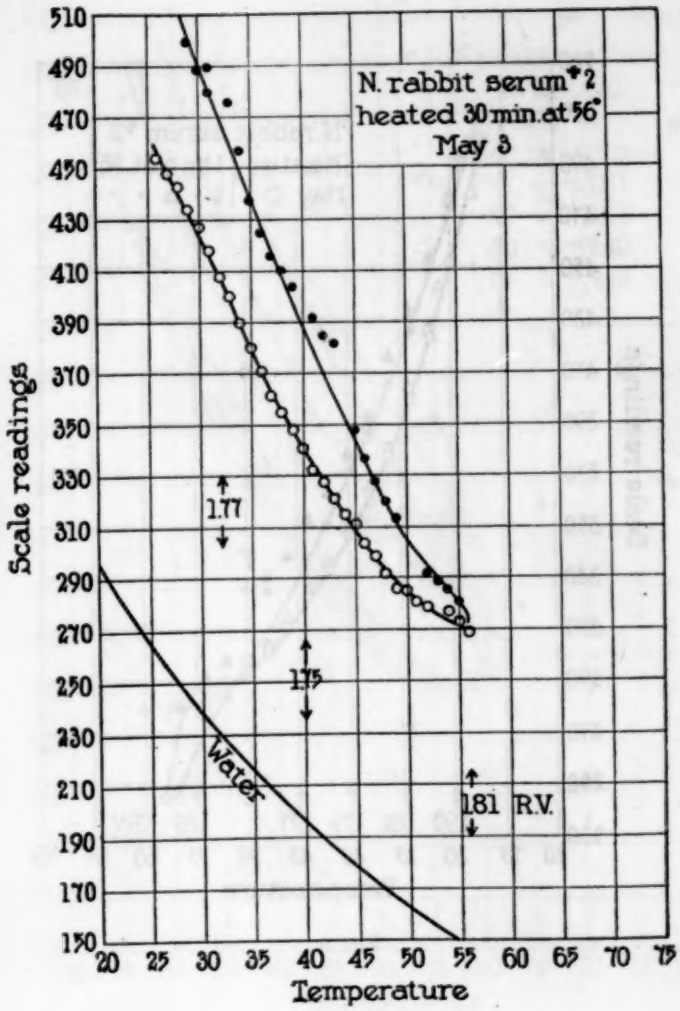
N. rabbit serum *2
heated 30 min. at 56°
May 3
Scale readings
Temperature
1.77
1.75
1.81 R.V.
Water
510
490
470
450
430
410
390
370
350
330
310
290
270
250
230
210
190
170
150
20
25
30
35
40
45
50
55
60
65
70
75

FIG. 5.

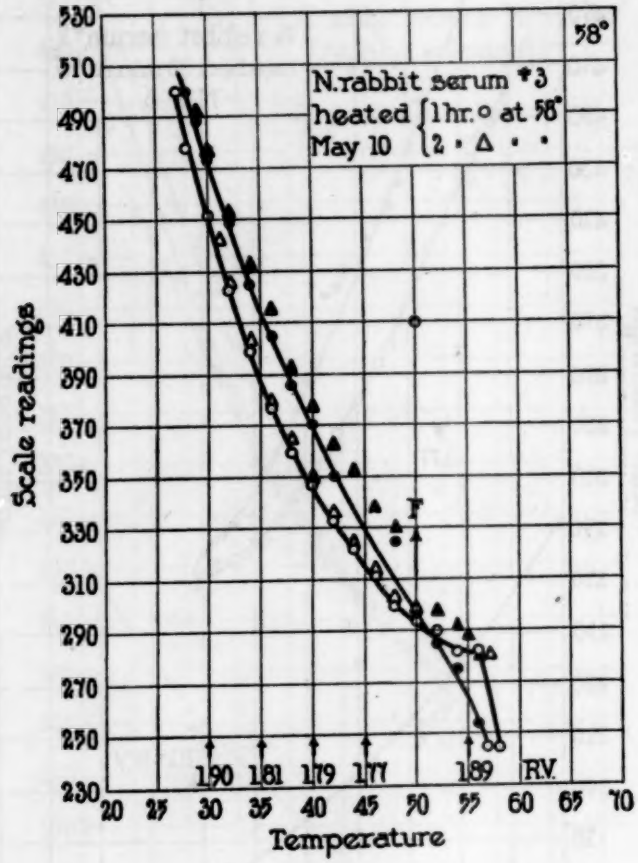
58°
N.rabbit serum *3
heated { 1 hr. ○ at 58°
May 10 { 2 • Δ • •
Scale readings
530
510
490
470
450
430
410
390
370
350
330
310
290
270
250
230
1.90
1.81
1.79
1.77
1.89
RV.
20 25 30 35 40 45 50 55 60 65 70
Temperature

FIG. 6.

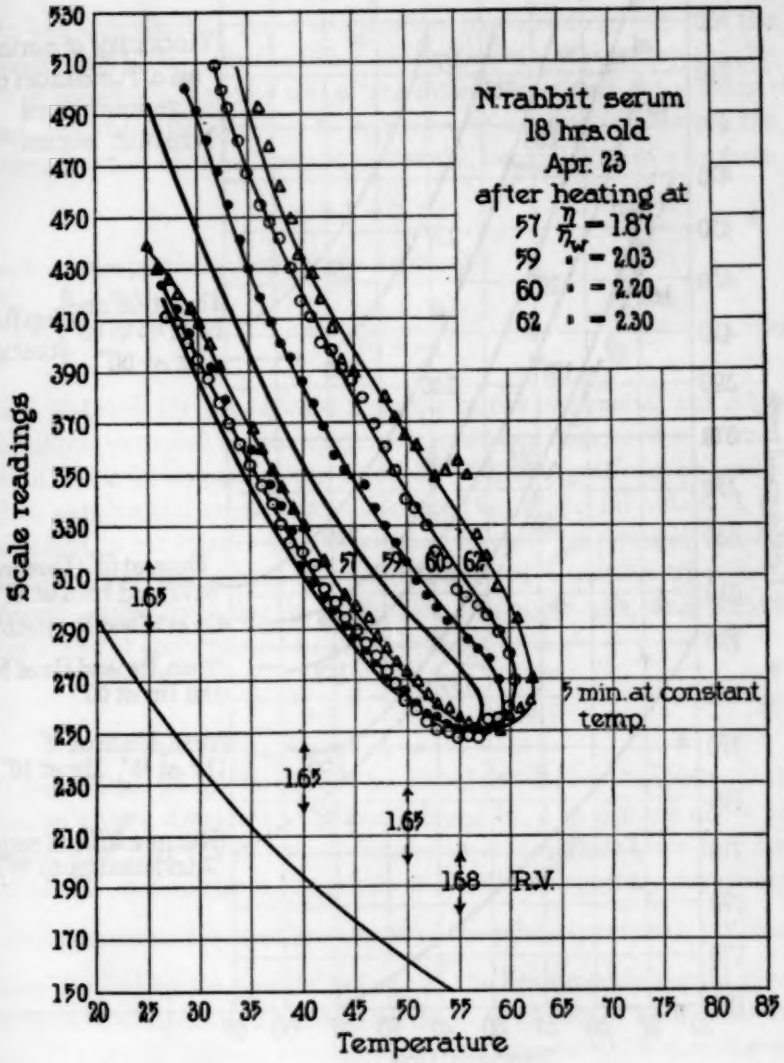
N.rabbit serum
18 hrs.old
Apr. 23
after heating at
57 η/η_w = 1.87
59 " = 2.03
60 " = 2.20
62 " = 2.30
5 min.at constant temp.
1.65
1.65
1.65
1.68 R.V.
57
59
60
62
Scale readings
Temperature

FIG. 7.

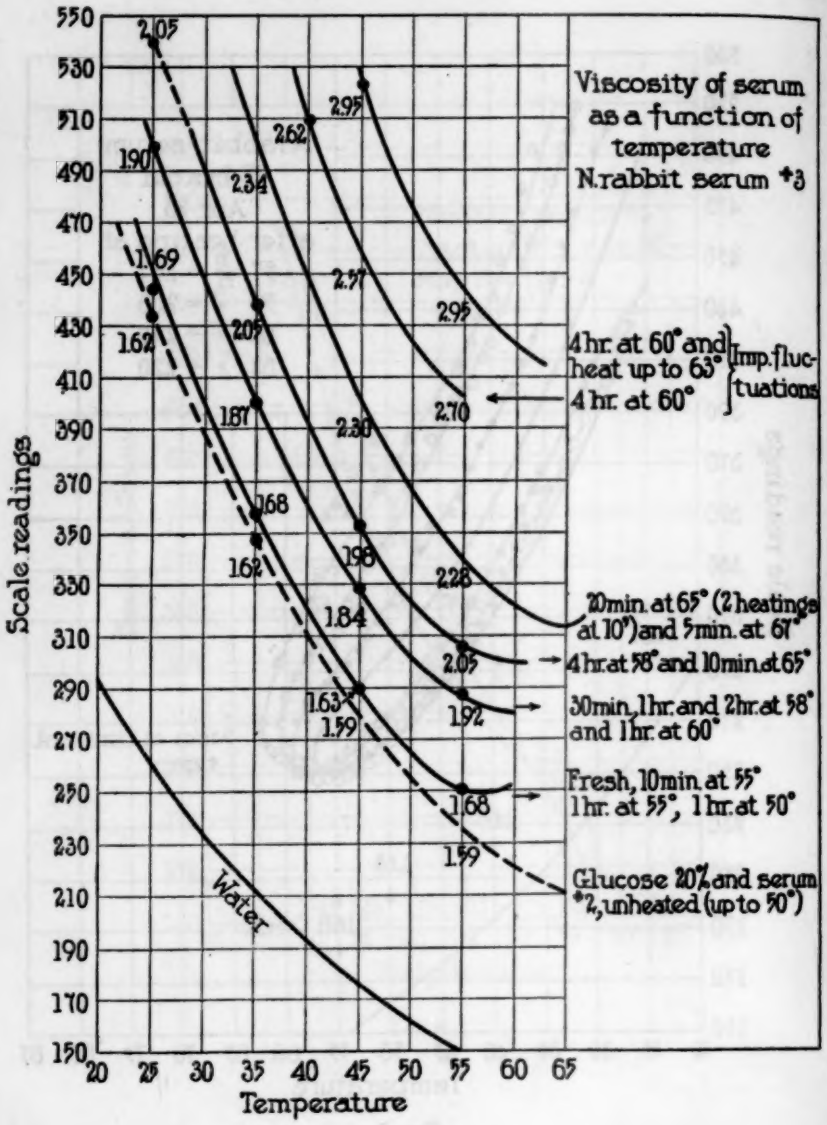
Viscosity of serum as a function of temperature
N. rabbit serum *3
4 hr. at 60° and heat up to 63°
Imp. fluctuations
4 hr. at 60°
20 min. at 65° (2 heatings at 10') and 5 min. at 61°
4 hr. at 58° and 10 min. at 65°
30 min., 1 hr. and 2 hr. at 58° and 1 hr. at 60°
Fresh, 10 min. at 55° 1 hr. at 55°, 1 hr. at 50°
Glucose 20% and serum *2, unheated (up to 50°)
Water
Scale readings
Temperature
2.05
1.90
1.69
1.62
2.34
2.62
2.95
2.77
2.95
2.70
2.09
1.87
2.30
1.68
1.62
1.98
2.28
1.84
2.05
1.63
1.59
1.92
1.68
1.59

FIG. 8.

DISCUSSION.

In our experiments the concentration remains constant, yet the viscosity increases. On the other hand, Einstein[2] states that the degree of dispersion, *i.e.*, the size of the molecules or particles is immaterial, and that viscosity can be expressed as a linear function of the volume fraction of the dispersed substance, according to the formula

$$\eta = 1 + 2,5\,\varphi \tag{1}$$

which is an approximation of the expression

$$\eta = \frac{1 + 0.5\,\varphi}{(1 - \varphi)^2} \tag{2}$$

where η stands for the relative viscosity of the suspension, and φ for the volume occupied by the dispersed substance expressed as a fraction of the total volume of the solution. These equations, however, fail to express the experimental facts. The reason for this lack of agreement is not clear. But Kunitz has recently proposed an empirical formula which applies remarkably well to a number of widely different cases, including lyophilic and lyophobic sols, and within large limits of concentration. This formula is[3]

$$\eta = \frac{1 + 0.5\,\varphi}{(1 - \varphi)^4}. \tag{3}$$

The problem we are facing can be stated as follows: Assuming that Einstein's view concerning the part played by φ as defined above is correct, and that the degree of dispersion is immaterial, we can use formula (1), and compute the values of φ, and consequently determine $\frac{\varphi}{C}$ which expresses the specific volume of the solute (proteins). These values divided by the specific volume of the dry proteins should give the amount of hydration of the proteins in solutions, at temperatures up to 55°, and its increase as a function of temperature. But we know that this formula does not apply to lyophilic sols at high concentration, consequently, we cannot rely on the figures at all.

[2] Einstein, A., *Ann. Physik.*, 1906, xix, 289; 1911, xxxiv, 591.
[3] Kunitz, M., *J. Gen. Physiol.*, 1926, ix, 715.

On the other hand we can apply Kunitz's formula (Table I) which we know fits the experimental facts very satisfactorily but then the main assumption of Einstein concerning the rôle of the degree of dispersion, which is a consequence of his mathematical derivations, may not hold any longer. Therefore, it does not seem possible, at present, to decide whether the figures computed in this way express quantitatively the increase due to hydration alone or whether some other

TABLE I.

Viscosity of Rabbit Serum 3, Heated at Different Temperatures, and Values of φ *and* $\frac{\varphi}{C}$ *Computed from Kunitz's formula (see Figs. 7 and 8).*

C = mean concentration of proteins in the serum = 6.5 per cent.

1	2	3	4	5	6	7
Relative viscosity $\frac{\eta}{\eta_0}$	φ	Specific volume $\frac{\varphi}{c}$	Specific volume of dry proteins	Ratio Column 3 / Column 4	Increase per cent of specific volume (Hydration ?)	Increase in hydration due to heating
	per cent				*per cent*	*per cent*
1.65	10.7	1.645	0.785	2.09	109	Unheated
1.69	11.2	1.720	"	2.19	119	
1.88	13.3	2.030	"	2.58	158	39
2.03	14.8	2.275	"	2.90	190	71
2.30	17.2	2.650	"	3.37	237	118
2.62	19.8	3.050	"	3.88	288	169
2.95	21.8	3.350	"	4.27	327	208

phenomenon is also responsible for it to a certain extent. However, column 6 has been tentatively designed as "hydration."[4]

[4] The influence of the charge of colloidal particles on the viscosity of the sol has been taken in consideration, especially by von Smoluchowski (*Kolloid-Z.*, 1916, xviii, 194.) who enlarged Einstein's formula which became:

$$\eta_s = \eta_M \left\{1 + 2.5\,\varphi \left[1 + \frac{1}{\lambda\,\eta_M\,r^2}\left(\frac{D\,\zeta}{2\,\pi}\right)\right]\right\}$$

however our results are not in accord with this formula, which should lead to a lower viscosity as the size of the particles increases. We observe the contrary, and there seems to be no doubt that after heating the size of the particles increases, since coagulation is the limit of the phenomenon. Perusal of Freundlich's excellent discussions of the subject in general (Colloid and capillary chemistry, New York, pp. 367 and following; 539, etc.) will repay the reader.

All that can be said is that it expresses the increase per cent of the specific volume of the serum proteins. It is interesting to note that the figures expressing "hydration" obtained from Einstein's formula (3) are larger (3.71 times) than those obtained from Kunitz's formula (1), and that this ratio remains constant up to a viscosity of 2.03. From this figure up the discrepancy begins and increases rapidly with increasing viscosity.

Fig. 8 suggests another observation: the dotted curve (viscosity 1.62) was obtained with a sugar solution (glucose 20 per cent). The concentration is 3 times that of the serum. Hence, serum may be said to behave exactly like a true solution, as a function of temperature, up to nearly 55°. It is surprising that such a concentrated solution of proteins should have such a low viscosity. When, by diluting the serum, its specific volume is made equal to that of the sugar solution, its viscosity is much inferior to that of the latter.

FRACTIONATION OF GELATIN.

By M. KUNITZ and JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Accepted for publication, July 2, 1928.)

It has been shown by the writers[1] that such phenomena as the swelling of electrolyte-free gelatin blocks, the high viscosity of gelatin solutions, the decrease of the pH effect on viscosity with increase in the concentration of gelatin solutions, and, finally, the peculiar phenomenon of shrinking of dilute gels when placed in cold water, are explainable on the assumption that gelatin consists of a mixture of at least two substances, one of which is soluble in cold water, while the other is insoluble even in warm water. Actually, it has been found possible to isolate from gelatin by means of alcohol precipitation two fractions which differ enormously in their physical properties.[2] One of the fractions has properties similar to those of albumin; it is soluble even in ice water, does not set to a gel, has a low viscosity, and does not give rise to the pH-viscosity curve characteristic for gelatin. Besides, it has an osmotic pressure at its isoelectric point (pH 4.7) considerably higher than that of isoelectric gelatin, from which it has been isolated. The second fraction has properties opposite to those of the first one; it is insoluble in cold water, sets to a gel at a low concentration, swells much less, and has a higher viscosity and lower osmotic pressure than ordinary gelatin. But this fraction is still "soluble" in warm water.

It had been previously found by Schryver[3] and his associates that a soluble material could be obtained from gelatin by allowing isoelectric salt-free gelatin to stand at 14–16°C. Under these conditions the

[1] (*a*) Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926–27, x, 161. (*b*) Kunitz, M., *J. Gen. Physiol.*, 1926–27, x, 811. (*c*) Northrop, J. H., *J. Gen. Physiol.*, 1926–27, x, 893. (*d*) Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926–27, x, 905. (*e*) Kunitz, M., *J. Gen. Physiol.*, 1928–29, xii, 289.

[2] Northrop and Kunitz,[1a] p. 167.

[3] Knaggs, J., Manning, A. B., and Schryver, S. B., *Biochem. J.*, 1923, xvii, 473.

gelatin separates out as a clot leaving some soluble protein material in the supernatant fluid.[4] If the precipitate is then suspended in water a lower concentration of the soluble material is found in the supernatant solution. This solution moreover did not have the properties of ordinary gelatin, so that, as these workers point out, it can not be considered as a saturated solution of gelatin. These experiments were repeated and confirmed by the present writers, but it was found that if the gelatin were allowed to stand at 23° instead of 15° a very much larger amount of the soluble material was obtained and this fact was used as the basis of the method of separation used in the present paper.

Experimental Procedure.

A stock of isoelectric gelatin is prepared from Cooper's non-bleached gelatin by the modified Loeb's method as described elsewhere.[6] A solution of 60 gm. air-dried isoelectric gelatin (85 per cent dry weight) is made in 3000 cc. of distilled water, which has been adjusted to pH 4.7 by means of acetic acid, filtered through cotton wool into Pyrex flasks, and put into a water bath kept at a constant temperature of 23°C. A layer of toluene is added to the gelatin to prevent mould and bacterial growth. The gelatin solution gradually turns milky white, thickens, and after several days the thick white gel begins to break up into a gelatinous precipitate which settles slowly. The precipitation may be accelerated by stirring up the gel occasionally. After 5 or 6 days the flasks are removed from the bath, the supernatant solution is decanted as much as possible, and the rest is centrifuged, care being taken not to heat the gelatin in the centrifuge. The centrifuged precipitate is stirred up once or twice in the centrifuge bottles with cold water of pH 4.7 and recentrifuged. It is then melted and made up to about 80 per cent of the original volume of the 2 per cent solution with water of pH 4.7, and allowed to set at 23°C. for the second precipitation. The process is repeated 12 to 15 times until a precipitate is left which does not dissolve even at 50°C. Care has to be taken during the whole process that the pH should remain at 4.7. The final precipitate is washed several times in the centrifuge bottles with hot water, and transferred into 95 per cent alcohol, where it is allowed to remain overnight. The alcohol is then decanted off and the gelatin is ground up in a mortar with ether, which is allowed finally to volatilize.

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[4] Schryver and Thimann[5] were also able to obtain a soluble and insoluble fraction by means of electrolysis of gelatin solutions.

[5] Schryver, S. B., and Thimann, K. V., *Biochem. J.*, 1927, xxi, 1284.

[6] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1927–28, xi, 477. Gelatin prepared in this way is practically free from diffusible nitrogenous material, *cf.* Knaggs, Manning, and Schryver.[3]

The air-dried precipitate is a fine powder of a light chocolate appearance. The yield is about 1 per cent of the original amount of gelatin. The supernatant solutions may be used for preparation of the soluble fraction by means of alcohol, as described elsewhere.[2] If the precipitation is carried out at a lower temperature than 20°C. practically

TABLE I.

Precipitation of Isoelectric Gelatin at 23°C.

	Volume	Dry gelatin	Strength of solution
	cc.	*gm.*	*per cent*
Original	3000	51	1.66
1st precipitate	2100	43.5	2.07
1st supernatant solution	900	6.9	0.77

TABLE II.

Influence of Initial Concentration of Gelatin Solution on Concentration of Gelatin in the Supernatant Liquid after 1st Precipitation at 23°C.

Initial concentration in gm. dry gelatin per 100 gm. solution	0.6	1.17	1.70	2.35	2.93
Gm. dry gelatin per 100 gm. supernatant liquid	0.19	0.53	0.68	1.21	1.19

TABLE III.

Gelatin Found in Supernatant Liquid on Repeated Precipitation of a Gelatin Solution at 23°C. Original Amount of Gelatin 51 Gm. by Dry Weight.

Precipitation	1	2	3	4	5	6	7	8
Concentration of gelatin in supernatant liquid in gm. per 100 cc. solution	0.77	0.55	0.48	0.50	0.50	0.40	0.37	0.42
Gm. gelatin in the supernatant liquid	6.9	9.5	5.6	1.2	3.1	4.7	2.3	1.9
Precipitation	**9**	**10**	**11**	**12**	**13**	**14**	**15**	
Concentration of gelatin in supernatant liquid in gm. per 100 cc. solution	0.31	0.31	0.24	0.15	0.15	0.15	0.12	
Gm. gelatin in the supernatant liquid	1.9	2.0	1.5	0.9	0.98	0.61	0.54	

all of the gelatin comes out as a precipitate with only a trace remaining in the supernatant solution. On the other hand, when the precipitation is done at 23°C. 14 to 18 per cent of the original gelatin remains in the supernatant solution. The distribution of gelatin between the precipitate and the supernatant solution after the first precipitation at 23°C. is shown in Table I.

The concentration of soluble material increases with the total concentration of gelatin, as shown in Table II.

On further precipitation the amount of gelatin in the supernatant solution gradually decreases, as shown in Table III.

The Physical Properties of the Separated Fractions of Gelatin.

Solubility.—The difference in the solubility of the various fractions of gelatin appears even after the first precipitation. Thus, in one of the experiments a solution of the first precipitate and of the first supernatant solution was completely precipitated by means of alcohol, washed with ether, and dried in the air. Suspensions were then made of 0.1 gm. dry powder of the two fractions in 10 cc. of water each, at 25°C. The fraction from the supernatant solution dissolved immediately in the water producing a clear solution, while the fraction from the first precipitate swelled but did not dissolve unless it was heated. After repeated precipitation the solubility of the precipitate becomes less until finally it does not dissolve even in hot water. The insoluble fraction appears only on repeated fractionation. Simple reheating of gelatin without separation of the precipitate does not produce any visible effect on the gelatin. A 2 per cent solution remained unchanged after heating to 50° and cooling to 23° more than 20 times during a period of 2 months.

Swelling of Powdered Gelatin.

The final precipitate does not swell when suspended in cold water. Its bulk is the same whether it is suspended in water, alcohol, or toluene. This is shown in Table IV. The bulk was determined by centrifuging suspensions of 0.1 gm. of the dry powder in 10 cc. of various liquids to constant volume of sediment. As a comparison suspensions were also made of ordinary isoelectric gelatin which had been precipi-

tated and dried by means of alcohol and ether in the same way as the fractionated gelatin, and also of the precipitate from the first precipitation.

Thus it is seen that when 0.1 gm. of ordinary gelatin is suspended in water it has a bulk of 0.9 cc. when centrifuged as compared with the bulk of 0.25–0.30 cc. when it is suspended in absolute alcohol or toluene; on the other hand, the insoluble fraction of gelatin from the final precipitation has practically the same bulk, 0.35–0.40 cc., no matter whether water, alcohol, or toluene is used. It is to be noted that the insoluble gelatin which had been precipitated only once gives higher swelling in water than ordinary gelatin, which is partly soluble at 25°C.

TABLE IV.

Comparison of Swelling of Various Types of Gelatin at 25°C.

Liquid	Insoluble gelatin from final precipitation	Insoluble gelatin from first precipitation	Normal isoelectric gelatin
	Cc. sediment		
Distilled water	0.40	1.20	0.90
Absolute alcohol	0.35	0.20	0.30
Toluene	0.35	0.25	0.25

Effect of Acid or Alkali on the Swelling of the Insoluble Fraction of Gelatin.

Addition of dilute HCl or NaOH to a suspension of the insoluble fraction of gelatin in water brings about a considerable swelling of the particles of gelatin, owing to the unequal distribution of the diffusible ions between the particles and the outside solution in accordance with the Donnan equilibrium. The swelling increases with increase in the amount of acid or alkali until a pH about 3.0 on the acid side or 9.0 on the alkaline is reached when the material begins to dissolve on heating, forming a viscous solution. On bringing the solution back to pH 4.7 the material rapidly precipitates out in a flocculent state.

Swelling of Gels Made of Mixtures of the Soluble and the Insoluble Fractions of Gelatin.

The mechanism of swelling of gels of isoelectric gelatin has been studied extensively by the writers.[1] The final conclusions reached may

be summarized as follows. There are three forces which affect the swelling of isoelectric gels:

1. The osmotic force due to the presence of a soluble ingredient of gelatin even at temperatures as low as 5°C. This force causes the block of gel to take up water, and is the significant factor in gels of high gelatin content.

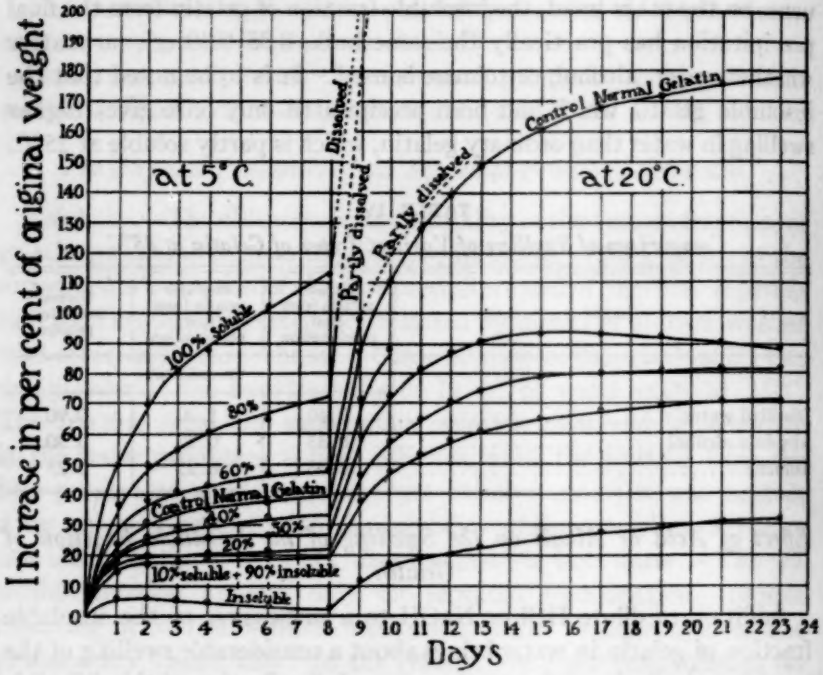

FIG. 1. Swelling of 30 per cent gels consisting of mixtures of insoluble and soluble fractions of gelatin. Blocks of gel weighing about 1.5 gm. each were allowed to swell in 200 cc. M/30 acetate buffer pH 4.7.

2. The residual elastic force in the micellæ, which originates while the gelatin is still in the sol state. This force causes the block of gel to lose water and shrink. It is the significant factor in dilute gels.

3. Finally the elastic force of the block of gel as a whole which resists both the swelling and the shrinking of gels.

According to this viewpoint a concentrated gel made up entirely of the insoluble ingredient should not swell at all in the absence of acid,

alkali, or salt, since there are no soluble molecules to produce an osmotic force. Addition of increasing amounts of the soluble fraction to a gel of the insoluble fraction of gelatin gives rise to osmotic pressures, and increased swelling should be expected. Fig. 1 shows what actually takes place when gels containing various mixtures of the insoluble and soluble ingredients were placed in a M/30 acetate buffer pH 4.7. The experiment was done as follows:

A series of solutions containing 30 gm./100 gm. of solvent was made up of various proportions of soluble and insoluble fractions in warm 0.075 N NaOH. 2 cc. of each solution was poured into a mould of glass tubing, mounted on a paraffin block, and allowed to set for 24 hrs. in the refrigerator at 5°C. The blocks of gel were then removed from the glass tubing, weighed, and put into 200 cc. of M/30 acetate buffer pH 4.7 which had been previously cooled to 5°C. The blocks were weighed at various intervals of time in a refrigerating room which was kept at constant temperature of 5°C. A block of 30 per cent ordinary isoelectric gelatin dissolved in 0.075 N NaOH was prepared and put into 200 cc. of M/30 acetate buffer pH 4.7 as a control. The blocks remained at 5°C. for 8 days, and then were transferred with the outside solutions to a constant temperature water bath of 20°C.

The plotted curves for the rate of swelling of the gels show that at 5°C. the gel consisting of 100 per cent insoluble fraction of gelatin swells slightly if at all, while the addition of the soluble ingredient brings about swelling. The swelling increases rapidly as the proportion of the soluble material increases. Gels consisting of more than equal parts of soluble and insoluble fractions swell more than the normal gelatin control. When transferred to 20°C. all gels begin to swell faster, and those that have been swelling more than the control at 5°C. swell at 20°C. so rapidly that in a day or so they dissolve completely leaving a residue of insoluble gelatin. The gel consisting entirely of the soluble fraction melts immediately when the temperature is raised to 20°C. That the fractionation is not complete yet is shown by the fact that even the 100 per cent *insoluble* gel swells somewhat at 20°C. which indicates that there is still left in it some of the fractions which are soluble at 20°C. It is also possible that the presence of the electrolytes of the buffer affect the solubility of the gelatin as was found before in the case of the swelling of ordinary gelatin. The fractionation of the soluble fraction used in this experiment was purposely stopped at this

point so that a 30 per cent gel at 5°C. would be obtained. By means of further fractionation of the soluble ingredient with alcohol it is very easy to obtain a gelatin fraction which does not set even at 5°C. no matter how concentrated the gel is.

Negative Swelling of Dilute Gels of Insoluble Gelatin.

When gels containing less than 10 per cent gelatin are placed in cold water or dilute buffer pH 4.7 the gels not only do not swell but actually shrink and lose water. It has been shown by one of the writers[1] that the force causing the dilute gels to lose water is the elasticity in the micellæ of gelatin. This elastic force, which is brought about by the hydration of the micellæ in the gelatin solution, is due, first, to the presence of soluble gelatin inside of the micellæ and, second, to an excess of diffusible ions inside of the micellæ over the outside solution. The osmotic pressure in the micellæ is therefore higher than in the surrounding solution and water enters until the elastic force is equal to the osmotic pressure. When the gelatin solution sets to a gel the soluble material precipitates and the ions diffuse out when the gel is placed in water. The osmotic pressure is thereby destroyed and the water is forced out by the elasticity of the micellæ, thus bringing about a gradual shrinking and loss of water by the block of gel as a whole. This contraction is greater the greater the amount of water originally held by the micellæ. The amount of water held in them is large in gelatin sols made up in acid or alkali. In ordinary gelatin the micellæ form only a part of the total gelatin, the rest of which is found in solution outside of the micellæ. A solution of the *insoluble* fraction of gelatin in dilute NaOH is not a perfect solution but rather a colloidal suspension of swollen micellæ, as shown by the high viscosity of the mixture. The number of micellæ per gm. of substance then is greater in the case of the insoluble fraction of gelatin than in the case of ordinary gelatin. Hence it is to be expected that when dilute gels are made up of both materials the one consisting of the insoluble component of gelatin should lose more water when put in dilute buffer pH 4.7 than the gel which was made up from ordinary gelatin. That this is actually what happens is shown by the following experiment.

Two solutions were made up each containing 0.1 gm. gelatin + 2 cc.

M/10 NaOH. One was made up of ordinary isoelectric gelatin, while in the other the insoluble fraction was used. Both solutions were allowed to set for 24 hours at 5°C. and then the gels were weighed and each put into 250 cc. M/30 acetate buffer pH 4.7 at 5°C. where they remained for about a month. The weight of the gels was determined at various intervals of time. Fig. 2 shows the results. It is seen that while the block of gel which was made up of ordinary gelatin has lost finally about 22 per cent of its original weight, the one that was made up of the insoluble fraction has lost as much as 68 per cent under the same conditions. The dry weight of the gel was unchanged so that the result is not simply due to solution.

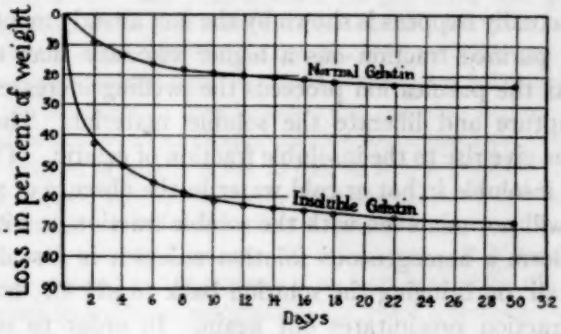

FIG. 2. Negative swelling of 5 per cent gels in M/30 acetate buffer pH 4.7 at 5°C.

The Irreversibility of Gelatin Fractionation.

According to the mechanism of swelling outlined above the soluble ingredient of ordinary gelatin is present both inside and outside of the micellæ. Each micella may be compared to a cell, filled with soluble gelatin; the cell wall, which is impermeable to the soluble gelatin, consists of the insoluble fraction of gelatin. The number of micellæ in a gelatin solution increases with the decrease in temperature until the setting point is reached. When a dilute solution of salt-free isoelectric gelatin is left at a temperature of about 23°C. the micellæ together with the contained soluble fraction of gelatin in them gradually settle down as a gelatinous precipitate, while the supernatant liquid consists of a

solution of practically pure soluble material. The process of removal of the soluble fraction of gelatin from inside of the micellæ is a difficult one owing to the impermeability of the micella wall. Only through a long series of reprecipitations accompanied by repeated heating of the solution is it possible to obtain an insoluble fraction which is apparently free from the soluble component. The liberation of the soluble material is probably due to a rupture of the micellæ by osmotic pressure. In a gelatin solution some soluble material exists both inside and outside of the micellæ. When the gelatin is allowed to set in dilute solution some of the soluble material escapes and is removed. On remelting the gelatin the osmotic pressure difference between the inside and outside of the micellæ is greater than before and they therefore swell more. That this actually happens is shown by the fact already mentioned that a partially purified fraction has a higher viscosity than the orginal gelatin. As the purification proceeds the swelling increases until the micellæ rupture and liberate the soluble material. The ruptured micellæ thus give rise to the insoluble fraction of gelatin. This fraction is not only insoluble in hot or cold water in the absence of acid or alkali, but it will not mix even with the soluble fraction or with ordinary gelatin to form a homogeneous solution unless it is first dissolved in acid or alkali; on bringing the solution back to pH 4.7, however, the insoluble fraction precipitates out again. In order to resynthesize gelatin from its final fractions it is not enough to mix the fractions in the right proportions but it is necessary to reintroduce the molecules of the soluble material into the insoluble particles; or, in other words, to fill again the micellæ cells with a substance for which the cell wall is impermeable. This may be partially accomplished in the case of a solid block since the soluble material is held in the network of micellæ forming the block but it has not been possible to introduce the soluble material into the individual micellæ. It is probable that in the manufacture of gelatin the soluble material is actually formed inside the micellæ.

It could be assumed that the soluble fraction was formed from the gelatin by an irreversible chemical reaction and hence mixing the fractions would not give the original gelatin. This assumption appears improbable since, as was shown, repeated heating and cooling does not result in the separation of the two fractions. It has also been found by

Alsberg and Griffing[7] that gelatin becomes partly soluble in cold water after prolonged grinding. If the grinding were sufficient to rupture the micellæ, the soluble material would be liberated.

Isolation of an Insoluble Substance from Gelatin by Partial Acid Hydrolysis.

A substance similar in many respects to the insoluble ingredient of gelatin described above is obtained when a 5 per cent solution of gelatin is partially hydrolyzed in M/10 HCl at 90°C. for about 2 hours. At this stage the gelatin no longer sets when cooled. On neutralization of the solution a flocculent precipitate appears, which can be centrifuged off and washed with water. On drying with alcohol and ether it gives a light brown powder similar to the one obtained by fractional precipitation. The yield is about 0.2 per cent. The powder is insoluble in hot or cold water, and swells considerably on addition of acid or alkali to its suspension in water until it is finally dissolved, as is the case with the insoluble component of gelatin from fractional precipitation. The only difference between the two substances is their behavior with regard to setting. It has been mentioned before that when an alkaline solution containing 5 per cent or more of the insoluble fraction of gelatin is left to cool to 5°C. it gradually sets to a gel. This does not happen with the insoluble substance obtained by acid hydrolysis of gelatin. A solution of it in dilute NaOH is very viscous, but it does not set even at a concentration of 10 gm. per 100 cc. solution. It is possible that the setting of gelatin depends on the presence of an ingredient which still exists in the material obtained by fractional precipitation but which is destroyed on hydrolysis with acid.

SUMMARY.

1. It is possible to fractionate gelatin by means of reprecipitation at 23°C. of a salt-free solution of pH 4.7 into two fractions, one of which is soluble in water at any temperature, and a second one which does not dissolve in water even when heated to 80°C.

2. The proportion of the soluble fraction in gelatin is much greater than of the insoluble one.

3. The insoluble fraction of gelatin does not swell when mixed with water, but it does swell in the presence of acid and alkali which finally dissolve it.

4. Blocks of concentrated gel made by dissolving various mixtures of

[7] Alsberg, C. L., and Griffing, E. P., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 142.

the soluble and insoluble fractions of gelatin in dilute NaOH swell differently when placed in large volumes of dilute buffer solution pH 4.7 at 5°C. The gel consisting of the insoluble material shows only a trace of swelling, while those containing a mixture of soluble and insoluble swell considerably. The swelling increases rapidly as the proportion of the soluble fraction increases.

5. A 5 per cent gel made up by dissolving the insoluble fraction of gelatin in dilute NaOH loses about 70 per cent of its weight when placed in dilute buffer pH 4.7 at 5°C. A similar gel made up of ordinary gelatin loses only about 20 per cent of its weight under the same conditions.

6. It was not found possible to resynthesize isoelectric gelatin from its components.

7. An insoluble substance similar in many respects to the one obtained by reprecipitation of gelatin is produce on partial hydrolysis of gelatin in dilute hydrochloric acid at 90°C.

DARK ADAPTATION AND THE LIGHT-GROWTH RESPONSE OF PHYCOMYCES.

By E. S. CASTLE.

(From the Laboratory of General Physiology, Harvard University, Cambridge.)

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I.

It has long been known that certain plants respond to an increase in intensity of illumination by a brief increase or decrease in growth rate. The increase was first shown by Blaauw (1914) for the unicellular, light-sensitive sporangiophores of *Phycomyces*, and simultaneously the decrease by Vogt (1915) for the etiolated coleoptiles of *Avena*.

If a sporangiophore of *Phycomyces* growing vertically in the dark is illuminated from above, or laterally from a number of opposed sources of equal intensity, after a definite interval an increase in the vertical rate of growth is observed, later followed by a decrease and brief fluctuations until the original rate is regained (see Fig. 1). This response has been called by Blaauw the "light-growth" response. If the light is maintained, the sporangiophore which has regained its original growth rate is then insensitive to that particular light, or "light-adapted." In order to produce a new growth response the intensity of the light must be again increased by a definite amount. The inverse phenomenon of "dark-adaptation" is shown by an increase of sensitiveness to stimulation by light as the sporangiophore is kept in the dark.

In the absence of precise studies of the kinetics of photic excitation and adaptation in plants it is of interest to examine the course of dark adaptation in *Phycomyces*, particularly as in the sporangiophores of this form the whole mechanism of reception and reaction is contained within one cell wall, and is probably confined to a very small region of that cell. Thus Errera (1884) showed that the whole growing zone of

the sporangiophore of *Phycomyces* was within 1.0 mm. of the sporangium, and Blaauw (1918) that the light-sensitive region probably did not extend more than 2.0 mm. below it.

II.

Pure cultures of *Phycomyces blakesleanus Burgeff* ("+" strain) were grown in short glass vials as previously described (Castle, 1927–28). For experimentation a culture was placed in a moist glass cell with plane walls and cover nearly submerged in a water thermostat enclosed by a dark room. The temperature of the bath was held at 24.4°C. throughout, the atmosphere of the cell being saturated with water vapor at that temperature. By means of a sliding block either of two incandescent bulbs controlled by separate switches could be brought directly above the culture. One of low intensity was used as an initial adapting light, the other high intensity bulb being later pulled into position and used for brief stimulation. Heat was screened from the culture by an interposed plate of colloidal gold glass. The particular sporangiophore under consideration was observed laterally against an intermittent red light by means of a horizontal microscope which projected into the dark room enclosing the thermostat and contained an ocular micrometer scale against which elongations of as little as 10μ could be noted.

An actual experiment was run as follows: a culture placed in the observation cell was allowed to become thoroughly adapted thermally and photically for at least an hour, the low intensity lamp giving an intensity of illumination of 1.8 ft. candles. At an instant noted with a stop-watch the light was put out, and the culture allowed to remain in darkness for a measured interval. The 150 watt lamp, giving an intensity of 40 ft. candles, was now pulled into position and flashed on for 0.10 minute. Meanwhile and during the ensuing darkness the position of the sporangiophore, read to the sharp upper meniscus of the sporangium, was observed at intervals of $\frac{1}{4}$ minute. These micrometer readings when plotted against the time follow a nearly linear or slightly declining sequence until the growth response begins, when the points rise rapidly (Fig. 1). The first significant upward deviation, read to the nearest quarter of a minute, is taken as the time of the response.

The sporangiophore is then readapted to the low intensity by an exposure of 30 minutes, and restimulated after different times in the dark with the same quantity of light energy.

III.

Table I presents the average data for the reaction times of twenty sporangiophores, each figure being the mean of ten separate deter-

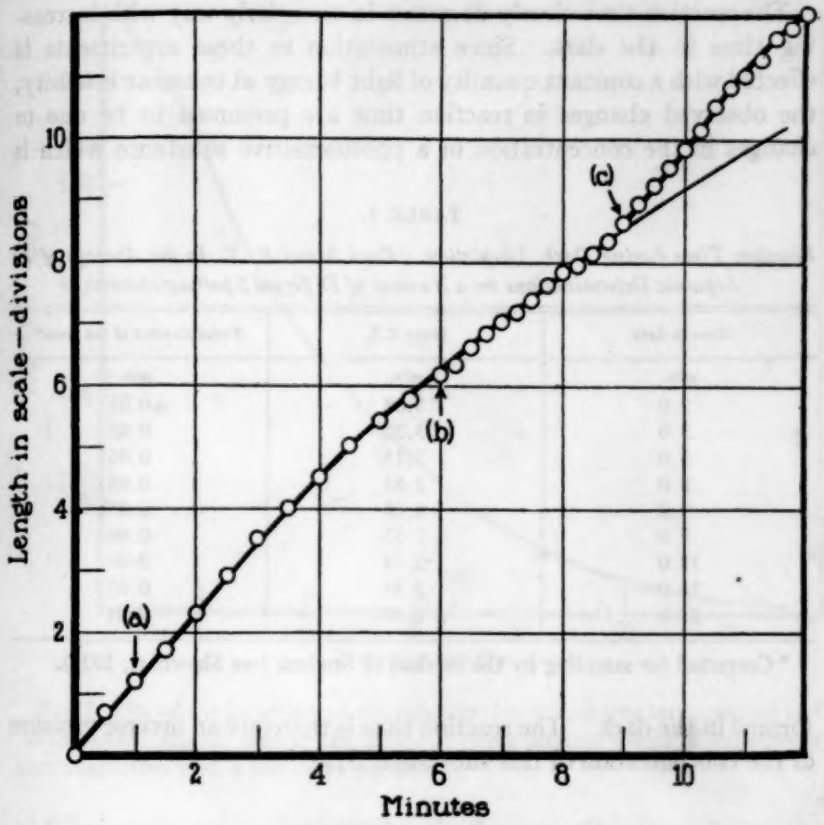

FIG. 1. The light growth response of *Phycomyces*. Sporangiophore previously growing in light of 1.8 ft. candles intensity, which is put out at (*a*). Dark adaptation of 5 minutes duration from (*a*) to (*b*). At (*b*) stimulation for 0.10 minute with 40 ft.candles. First increase in growth rate at (*c*), 3.0 minutes after stimulation. The slight falling off in rate during dark adaptation is not a random variation and usually occurs if the time in the dark is long enough. On the figure the trend of the growth curve previous to the response is continued as a thin line.

minations. Such averaging is justifiable, as seen from the small size of the corrected probable errors, in spite of the known individual

variability in absolute growth rate and in the temperature relations of growth (Castle, 1927–28). The photosensitive system must therefore possess a considerable degree of autonomy within the limits of the unicellular sporangiophore.

The reaction time clearly decreases in an orderly way with increasing time in the dark. Since stimulation in these experiments is effected with a constant quantity of light energy at constant intensity, the observed changes in reaction time are presumed to be due to changes in the concentration of a photosensitive substance which is

TABLE I.

Reaction Time during Dark Adaptation. Each Mean R. T. Is the Average of 10 Separate Determinations on a Number of Different Sporangiophores.

Time in dark	Mean *R.T.*	Probable error of the mean*
min.	*min.*	*min.*
2.0	3.58	±0.04
3.0	3.30	0.05
4.0	3.15	0.06
5.0	2.85	0.08
7.0	2.68	0.05
9.0	2.55	0.06
12.0	2.48	0.05
16.0	2.35	0.05
25.0	2.33	0.04

* Corrected for sampling by the method of Student (see Shewhart, 1926).

formed in the dark. The reaction time is therefore an inverse measure of the concentration of this substance, S, or

$$S = \frac{1}{R.T. - M}$$

where M is a constant representing the sum of the true latent period resulting as a consequence of photochemical action and any mechanical "action time" which may be present.

If the formation of S in the dark were a first order process, a plot of log $(R. T. - M)$ against time in the dark should give a linear relationship. M is taken as 2.30 minutes, a value which is most favorable, within the limits of choice, to the log plot. The linear relationship

does not hold, as is seen in Fig. 2, where the points fall on a curved line, perhaps with an inflection point.

Assuming $M = 2.10$ minutes and $R.T._{(t-0)} = 7.4$ minutes, values

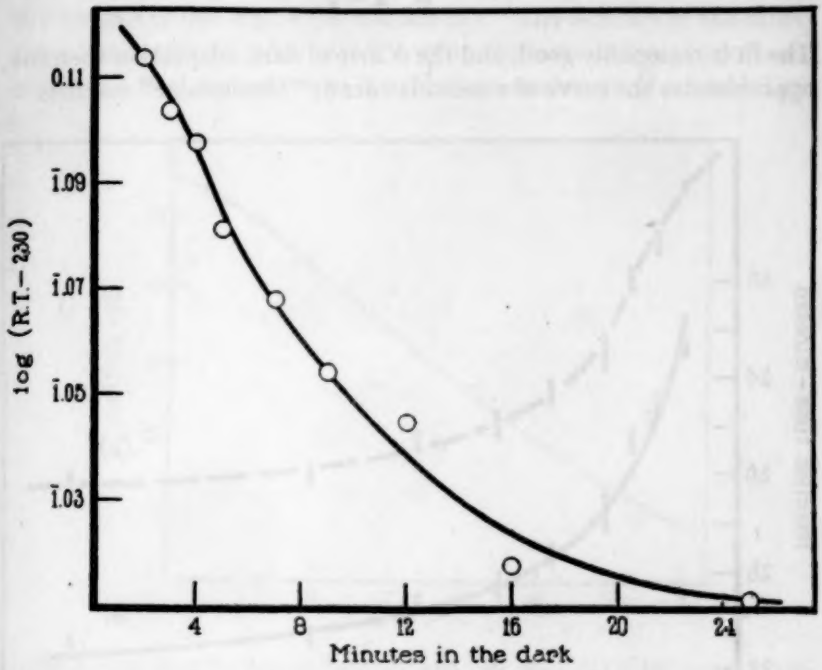

FIG. 2. Plot of the logarithm of the reaction time minus true latent period and "action time" against time in the dark. A linear relation would be expected if dark adaptation were a first order process.

of K have been calculated for pairs of adjacent points according to the formula for a bimolecular reaction

$$K(t_2 - t_1)\, a = \frac{x_2}{a - x_2} - \frac{x_1}{a - x_1}$$

where x stands for the amount of S formed. The average value of K is 0.012, and has been used to calculate the theoretical hyperbola

drawn through the observed points in Fig. 3 (*a*), by means of the formula

$$K = \frac{1}{at} \cdot \frac{x}{a - x}$$

The fit is reasonably good, and the course of dark adaptation therefore approximates the curve of a second order or "bimolecular" reaction.

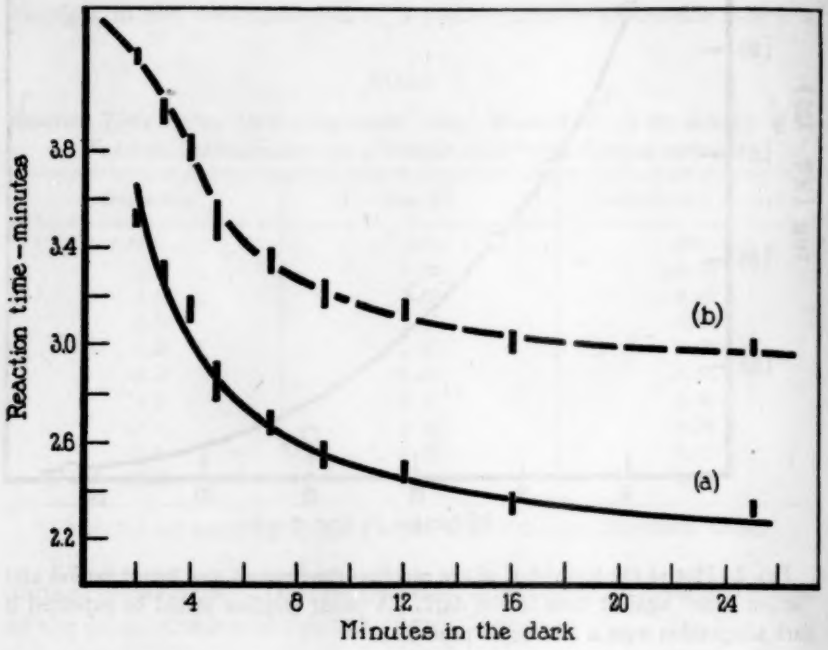

FIG. 3. Reaction time as a function of dark adaptation. In (*a*) the observed points are fitted with a hyperbola calculated according to the formula for a second order process. In (*b*) a free-hand curve is drawn through the same points, displaced on the ordinate scale, to illustrate the possible inflection of the curve. In each case the probable error of the mean reaction time is represented by one half the height of the symbol.

Crozier and Wolf (1928–29) studied the dark adaptation of *Agriolimax* during continuous photic excitation, and found the equations of a second order process with autocatalysis by the product of the reaction

necessary to account for the kinetics of the "dark" reaction. It is possible that an initial lag and subsequent inflection point in the curve relating reaction time and time in the dark such as would be expected on the basis of autocatalysis exists in the course of the dark adaptation of *Phycomyces* (see Fig. 3 (*b*) and Fig. 2). The location of the inflection point is at least not contradictory to the autocatalytic hypothesis, although a final decision as to the presence or absence of autocatalysis

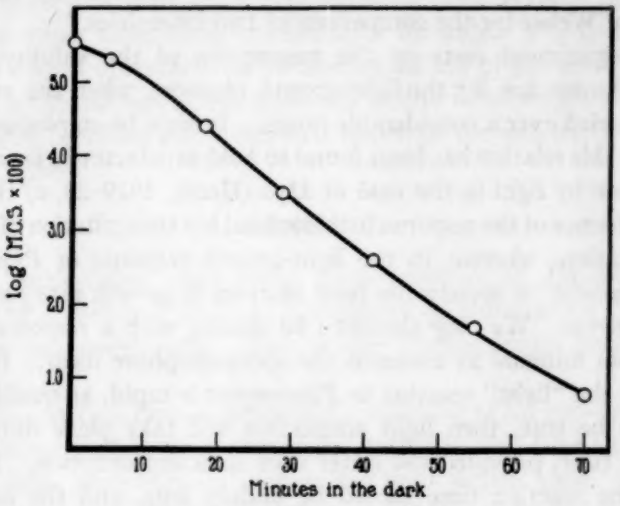

FIG. 4. Plot from the data of Tollenaar and Blaauw (1921) of the energy necessary to produce a just perceptible increase in growth rate after different times in the dark. (The time and intensity components of the energy product are not given in the original.)

cannot be made with the existing measurements. Another method of study of the photic adaptations of *Phycomyces* is at hand which is expected to render the distinction possible.

IV.

The only other attempt at a quantitative study of dark adaptation in plants has been made by Tollenaar and Blaauw (1921) on *Phycomyces nitens*. They determined the threshold sensitivity of sporan-

giophores by estimating the *quantity* of light necessary to produce a just perceptible increase in growth rate, following varying lengths of time in darkness after complete adaptation to light of 64 meter candles intensity. The logarithms of the energy products required to stimulate after different times in darkness are plotted in Fig. 4, taken from the data of Blaauw and Tollenaar, from which the authors conclude that the increase of sensitivity in the dark is a simple logarithmic function of the time in darkness, and that this relation "resembles the law of Weber for the comparison of two intensities."

This experiment rests on the assumption of the validity of the Roscoe-Bunsen law for the light-growth response, when the exposure time is varied over a considerable range. It must be emphasized that although this relation has been found to hold satisfactorily for minimal stimulation by light in the case of *Mya* (Hecht, 1919–20, *a*) the presence or absence of the response in this animal is a true criterion of threshold excitation, whereas in the light-growth response of *Phycomyces* the "threshold" is merely the least increase in growth rate *perceptible to the observer*. We may therefore be dealing with a response which is far from minimal as concerns the sporangiophore itself. In other words, if the "light" reaction in *Phycomyces* is rapid, as qualitatively seems to be true, then light adaptation will take place during the exposure time, provided the latter is of sufficient duration. In such a case the reaction time should be unduly long, and the complete differential for the production of S would have to be the sum of the partial differentials for the "light" and "dark" reactions. The validity of the Roscoe-Bunsen law for particular circumstances would not mean that the effectiveness of the light was at all times equivalent.

If we should assume that the curve in Fig. 4 represents the unopposed course of the "dark" reaction which regenerates S, the sensitive substance, it may be significant that there is an early inflection point as demanded by the notion of autocatalysis, and that the subsequent points do not fall on a straight line. In any case, the logarithmic relationship which might hold were Weber's law to be followed clearly does not exist, and since the data of Tollenaar and Blaauw give neither time nor intensity it is impossible to make with surety any quantitative inferences from the shape of the curve.

V.

It seems significant that the kinetics of dark adaptation in the unicellular sporangiophores of *Phycomyces*, where the mechanical aspects of the light response are so different from those in other forms, should prove to be strictly comparable to the other photosensory systems which have been studied, all of the latter being in animals: *Mya* (Hecht, 1918–19), the human retina (Hecht, 1919–20, *b*), and *Agriolimax* (Crozier and Wolf, 1928–29). In all of these systems the "dark" reaction which regenerates the sensitive substance is bimolecular, in spite of known differences in the specific nature of the photosensitive materials themselves. This similarity points to a certain identity in the *kind* of chemical mechanics that underlies the sensitivity of organisms to light.

SUMMARY.

1. A single-celled, elongating sporangiophore of *Phycomyces* responds to a sufficient increase in intensity of illumination by a brief increase in growth rate. This is the "light-growth response" of Blaauw.

2. The reaction time is compound, consisting of an exposure period and a latent period (this comprising both the true latent period resulting from photochemical action and any "action time" necessary for the response). During the latter period the plant may be in darkness, responding nevertheless at the end of the latent period.

3. Both light adaptation and dark adaptation occur in the sporangiophore. The kinetics of dark adaptation can be accounted for on the basis of a bimolecular reaction, perhaps modified by autocatalysis. Attention is called to the bimolecular nature of the "dark" reaction in all other photosensory systems that have been studied, in spite of the diversity of the photosensitive substances themselves and of the different forms of the responses to light.

I wish to acknowledge with thanks the suggestion by Dr. W. H. Weston, Jr. of the suitability of *Phycomyces blakesleanus* for the purposes of these experiments.

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HEME AND TISSUE IRON.

By M. L. ANSON and A. E. MIRSKY.

(From the Biophysical Laboratories of the Cancer Commission, Harvard University, Boston.)

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There are a variety of heavy metal compounds in living cells. These are supposed to be involved in the catalysis of biological oxidations. It is by combination with these heavy metal compounds that the respiratory poisons cyanide, hydrogen sulfide, and carbon monoxide are supposed to act.* Little has been known about the precise nature of these substances except that one cannot get a test for free ionic iron. Yet the properties of an iron compound, depend very much on the nature of the complex of which the iron is a part. Thus the iron in hemoglobin has not the properties of the iron in ferric chloride. Over 40 years ago, however, MacMunn (10) observed in a great variety of animal tissues a pigment complex related to heme, the iron pyrrol part of hemoglobin. Recently Keilin (6) has shown that this complex, which he calls cytochrome, is present in the aerobic tissues of both plants and animals generally and that it consists of a mixture of pigments allied to hemochromogen. The components of cytochrome are not identical with the hemochromogen prepared from hemoglobin. There are likewise other naturally occurring pigments, chlorocruorin, helicorubin, and actineohematin, which either are or can be converted into pigments similar to but not identical with the hemochromogen from hemoglobin.

The old view of hemochromogen was that it is simply reduced heme, the iron pyrrol complex of hemoglobin. On the basis of this view the various pigments which have been mentioned all contain different iron pyrrol complexes since they differ from each other and from the

*The reactions between the heme pigments and the specific inhibitors of respiration will be discussed in a later paper.

hemochromogen of hemoglobin. The writers (1, 3) have shown, however, that every hemochromogen consists of heme joined to some nitrogenous substance. The possibility thus arises that the various different hemochromogen-like pigments contain the same heme joined to different nitrogenous substances. When the unknown nitrogenous substances of helicorubin and actineohematin were replaced with pyridine or ammonia the resulting hemochromogens were identical spectroscopically with the pyridine or ammonia hemochromogens prepared from the heme of hemoglobin. So we concluded (2) that helicorubin and actineohematin contain heme. By the same method Fox (5) later showed that the iron pyrrol complex of chlorocruorin is different from heme. The addition of pyridine to yeast produced typical pyridine hemochromogen (2). This led us to suppose that cytochrome itself contained heme. But Keilin (7) has since presented evidence that there is in yeast in addition to cytochrome large amounts of invisible heme. Our pyridine hemochromogen may have come solely from this heme and not from cytochrome. This leaves the precise nature of the iron pyrrol nuclei of cytochrome and their relation to heme an open question. Nevertheless, the experiments based on the new view of the composition of hemochromogen permitted the identification of the heme of hemoglobin in aerobic tissues generally and they showed that in the study of the naturally occurring iron pyrrol pigments the ability of iron pyrrol groups to combine with nitrogenous substances must be taken into consideration. The significance of these results is emphasized by the recent experiments of Kuhn and Brann (9) and of Krebs (8) which show that the catalytic powers of heme are greatly changed when the heme is slightly altered or when it is combined with a nitrogenous substance.

The demonstration that heme itself is present in all animals makes less surprising the extraordinarily haphazard distribution of the hemoglobins. It would be interesting to know whether other substances containing metal pyrrol groups such as hemocyanin and chlorocruorin have, like the heme pigments, some ancestor common to all animals.

The fact that heme is universally distributed raises the question of how much of the iron of organisms is in the form of heme and how much in the form of still unknown iron compounds. In our original paper (2) we stated that we were attempting to answer this question

by parallel total iron and heme analyses, the heme being extracted with pyridine and estimated as pyridine hemochromogen. Our results have hitherto not been published because the validity of such a technique has become doubtful. In the meantime, however, several investigators (4, 11) have carried out heme estimations using essentially the same procedure we suggested. And von Euler and Fink (4) have come to the conclusion that, in the yeast they had, only about one 1/160 of the total iron is in the form of heme.

If pyridine and a reducer are added to yeast and the mixture first shaken and then centrifuged there is obtained on top a clear pyridine solution and on bottom a layer of yeast. When examined spectroscopically both these layers show hemochromogen-like bands. In the

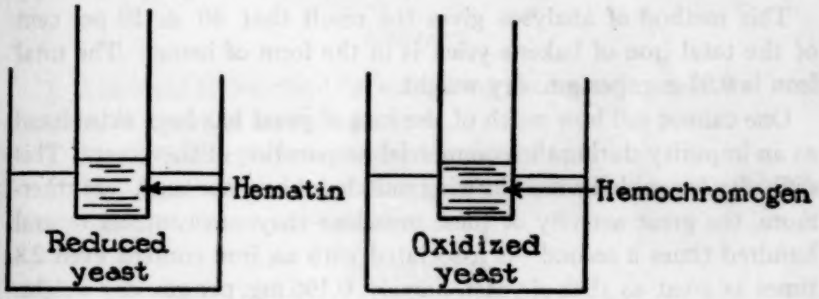

FIG. 1.

analyses of von Euler and Fink the pigment was examined in the clear pyridine solution and the assumption made that the concentration was the same in the yeast layer which was not examined. Such analyses are of little value in the absence of any precise information about the distribution of heme between the two layers. As a matter of fact, in the two cases we have studied, baker's yeast (Fleischmann's) and the wing muscles of bees, the assumption of equal distribution is not even approximately correct; mere inspection shows the concentration of hemochromogen to be much greater in the neglected yeast layer than in the clear pyridine solution.

Since we were unsuccessful in obtaining anything approaching a complete extraction of heme we decided to estimate the hemochromogen by comparing the density of its α band in the complete yeast-

pyridine mixture with the density of the α band of a series of known solutions of pyridine hemochromogen. In pure hemochromogen solutions whose concentrations are 10 per cent different one can readily detect the difference in the intensity of the bands. The hemochromogen from hemoglobin can be substituted for pyridine hemochromogen. If the heme contents of the solution of these two hemochromogens are the same, the intensity of the α bands are about the same too. In these experiments there were added to 4 gm. of moist yeast 1.5 cc. of 1/5 N NaOH and 1 cc. of pyridine.

It is necessary to compensate for the absorption of the yeast and its pigments. That is done as shown in the figure. By "oxidized yeast" (*cf.* Fig. 1) is meant yeast shaken with air for 5 minutes, which procedure causes the disappearance of the hemochromogen bands.

This method of analyses gives the result that 40 $\pm$ 10 per cent of the total iron of baker's yeast is in the form of heme. The total iron is 0.07 mg. per gm. dry weight.

One cannot tell how much of the iron of yeast has been introduced as an impurity during the commercial preparation of the yeast. This difficulty is avoided when the wing muscles of bees are used. Furthermore, the great activity of these muscles—they can contract several hundred times a second—is associated with an iron content even 2.8 times as great as that of yeast, namely, 0.196 mg. per gm. dry weight. Despite this great difference in the total iron we again found about 40 per cent of the iron to be in the form of heme.

The muscles were removed from frozen bees by the ingenious technique devised by Keilin (6). Care was taken not to introduce extraneous iron and to make the removal of the muscles as uniform as possible. Batches of the muscles from ten bees gave uniform analytical results.

The great weakness of these analyses lies in the use of pyridine hemochromogen as a standard. As Keilin (7) has pointed out, the hemochromogen-like substances obtained from cytochrome itself when pyridine is added may not be identical with pyridine hemochromogen. Furthermore, the α band of the hemochromogen seen in the muscle tissue obtained on centrifuging the muscle-pyridine mixture is not in precisely the same position as the α band of pyridine hemochromogen. These uncertainties render doubtful the estimations of heme based on the pyridine technique in any of its forms.

We hope, nevertheless, that the discussion and experiments which have been presented bring out some of the difficulties which have to be faced, and that they make clear, at least, that there is at present no adequate experimental basis for the view that only a very small part of tissue iron is in the form of heme. The evidence given by the type of procedure described in this paper points to heme being an important form of tissue iron.

The pyridine extract of yeast contains in addition to a hemochromogen similar to pyridine hemochromogen another hemochromogen-like pigment (2). Whether this second pigment contains an iron pyrrol complex is not known. There is the possibility that some and perhaps all of the iron not in the form of heme may exist in other iron pyrrol complexes.

SUMMARY.

1. A method is described for estimating the heme in yeast and bees' muscles as pyridine hemochromogen.

2. The difficulties of the method are discussed.

3. The heme as given by the pyridine method is responsible for about 40 per cent of the total iron.

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SPECTROPHOTOMETRIC STUDIES OF PENETRATION.

V. Resemblances between the Living Cell and an Artificial System in Absorbing Methylene Blue and Trimethyl Thionine.

By MARIAN IRWIN.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Accepted for publication, June 15, 1928.)

I.

INTRODUCTION.

This paper deals with an attempt to imitate the action of the living cell in respect to penetration of dyes.

The protoplasm of a living cell probably consists of two non-aqueous layers[1] (one in contact with the external medium and the other with the vacuolar sap), which are separated by an aqueous middle layer. But in setting up an artificial system we may confine ourselves to the simple case where the rate of diffusion appears to be controlled by three phases, as if a living cell consisted of only one non-aqueous layer lying between the external solution and the aqueous sap of the vacuole, as shown by the following diagram:

Aqueous sap in the vacuole
——— Vacuolar interface (v) ———
Non-aqueous layer of the protoplasm
——— External interface (e) ———
External dye solution

[1] We are unable to say which layer plays the more important rôle in regulating the rate of penetration into the vacuole. The controlling factor may change with alterations in the condition of the cells brought about by experiments. It seems possible that the external non-aqueous layer is more polar than the vacuolar layer, in that the ions penetrate the former more rapidly than the latter. In the case cited in the text the vacuolar non-aqueous layer may be responsible for the control of the rate. But in any case it seems probable that the net result may be treated as if the rate were controlled by a system containing only one non-aqueous layer.

In such a system the diffusion of a dye will be greatly affected by the partition coefficients:

$$K_e = \frac{\text{Concentration of dye in the non-aqueous layer}}{\text{Concentration of dye in the external solution}}$$

$$K_v = \frac{\text{Concentration of dye in the non-aqueous layer}}{\text{Concentration of dye in the aqueous sap}}$$

The higher K_e is, the more rapid is the rate of diffusion from the external solution through non-aqueous layer. On the other hand, the lower K_v is, the more rapid is the rate of diffusion through the non-aqueous layer. The values of K_e and K_v and concentrations of dye in the aqueous solutions will determine the diffusion gradient in the non-aqueous layer.

In the case of methylene blue, diffusion into the vacuole from the external solution is very slow because K_e is so small that though K_v is low there is very little dye in the non-aqueous layer[2] to diffuse into the sap. When a dye exists in two forms, with free base predominating at higher pH values and salt predominating at lower pH values, it is necessary to consider the partition coefficient of each form of dye at each interface. In the case of azure B the partition coefficient of the dye in the form of free base,[3] K_{eb}, is so high that its diffusion through

[2] According to Overton's theory penetration depends on the solubility of the dye in the lipoid layer of the living cell, which involves only one partition coefficient, K_e. This is sufficient to account for the lack of penetration of dyes which are insoluble in lipoid and for the rapid penetration of some of the dyes soluble in lipoid, but does not explain why a dye like crystal violet, which is very soluble in lipoid, does not readily penetrate the cell. On the basis of the theory presented in the text the slow penetration of crystal violet into the cell is explainable; though the dye penetrates the non-aqueous layer it does not enter the vacuole rapidly on account of K_v being so high.

[3] Though the dye in form of free base is often considered to be undissociated, this is an open question. The free base may be dissociated just as much as the dye in form of salt. We must therefore leave the question of the extent of dissociation of the "free base" for future solution by organic chemists, while we may conclude with more certainty that the "salt" is dissociated. This "free base" must be distinguished from the "pseudo base" which exists at still higher pH values. "Free base" may possibly be represented by an anhydro-base, or by a structure like the salt except that the halide is replaced by the hydroxyl group. (Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 927 and 928, foot-note 3.)

non-aqueous layer from the external solution is rapid. But even if $K_{\nu\flat}$ (the partition coefficient of the same form of dye at the vacuolar interface) is high, the dye readily diffuses through the non-aqueous layer into the vacuole because it is transformed to salt as soon as it comes in contact with the sap (on account of the low pH value of the sap) and this greatly increases the diffusion gradient. Since the partition coefficient of the dye in the form of salt at the vacuolar interface, $K_{\nu\nu}$, is low, its backward diffusion from the vacuole through non-aqueous layer is very slow. The dye salt readily collects in the vacuole. The same type of behavior occurs if a dye enters into combination with the sap to form a very slightly soluble compound.

This view would explain why azure B penetrates more rapidly than methylene blue into the vacuole of a living cell, as described in previous papers,[4-6] and would lead us to predict that the same thing would happen with an artificial system consisting of (1) the same dye solutions as those employed for the living cells, (2) chloroform to represent the non-aqueous layer of the protoplasm, and (3) sap freshly extracted from the vacuoles of living cells or artificial sap. Since the non-aqueous part of the protoplasm is but crudely represented by chloroform, the system may bear only a qualitative resemblance to the living cell.

If this theory[7] be correct we shall expect that when mixtures of dyes are employed the order of penetration will be the same in the artificial system as in the living cell.

II.

Methylene Blue.

A. Comparison of the Artificial System with Valonia.

Previous analyses[5,6] have shown that the vacuole of *Valonia* takes up azure B from a solution of methylene blue in sea water at pH 9.5. In order to compare this with the artificial system two types of

[4] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 425.

[5] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 927.

[6] Irwin, M., *J. Gen. Physiol.*, 1928–29, xii, 147.

[7] This theory has been successfully tested in the case of many dyes (Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 127; 1928, xxvi, 125).

experiments were made. (1) One experiment is to show the absorptive power of the chloroform in relation to the aqueous dye solution (representing the external dye solution used for living cells). This is done by first shaking the chloroform with the aqueous dye solution, allowing the chloroform to evaporate, and then dissolving the dye residue in the sap or in distilled water. The dye thus obtained is called for convenience "the dye obtained from chloroform by evaporation."

(2) The second experiment is to determine how readily the chloroform is able to give up the dye to the sap. This is done by first shaking the chloroform with the aqueous dye solution and subsequently extracting the dye from the chloroform by shaking it up with the sap.

If chloroform adequately represents the non-aqueous layer of a living cell we may by studying the behavior of the chloroform[8] toward the dye in these two aqueous phases learn something of the behavior of the non-aqueous layer of the cell in relation to phases inside and outside the cell.

The experiments were therefore carried out as follows.

200 cc. of solution of methylene blue (French) dissolved in sea water at pH 9.5 or at pH 5.5 was shaken up with 50 cc. of pure chloroform in a separatory funnel; the chloroform was then removed from the funnel and if it contained even a trace of water in the form of droplets the latter was removed by decanting the chloroform repeatedly from one beaker to another: the drops of water then adhered to the wall so that eventually chloroform free from drops of water could be obtained by this method. The chloroform was then divided into two equal parts: one portion was allowed to evaporate and the dye residue was dissolved in freshly extracted sap of *Valonia* (the sap contained about 0.6 M halides and had a pH value of about 5.8) or in distilled water. The second portion was shaken with sap until a concentration sufficient for spectrophotometric analysis was obtained. The sap was separated from the chloroform by pipetting it off (there was not sufficient chloroform in the sap to affect the absorption spectra of the dye in the sap).

The sample of methylene blue (French) gave an absorption curve with primary absorption maximum at 664 mμ when dissolved in sea water at pH 9.5 or pH 5.5 (Fig. 1, symbol □), which is characteristic of a dye solution consisting chiefly of methylene blue.

[8] Though the non-aqueous layer is represented by the chloroform, it does not signify that it resembles chloroform in chemical composition. It is uncertain as to whether the non-aqueous layers are "lipoid," but their behavior suggests it. The question of the character of the non-aqueous layers must therefore be left undecided for the present.

With this sample of methylene blue in sea water at pH 9.5, the dye (1) obtained from chloroform by evaporation, and (2) extracted from the chloroform by shaking with sap, gave an absorption curve characteristic of a dye consisting chiefly of azure B (with primary absorption

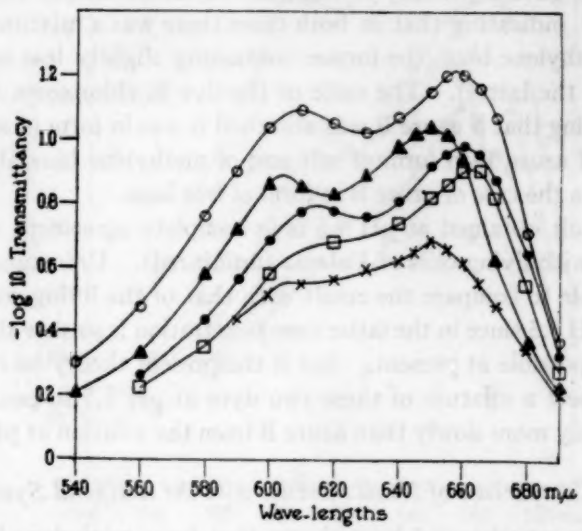

FIG. 1. Absorption curves obtained in experiments on methylene blue (French). (Comparison with *Valonia*.) Symbol □ represents the methylene blue dissolved in sea water at pH 5.5 or pH 9.5. Symbol ▲ represents the dye taken up by chloroform from methylene blue in sea water at pH 9.5, then freed from chloroform by evaporation, and finally dissolved in distilled water. Symbol × represents the dye extracted by sap of *Valonia* from chloroform which had previously been shaken with methylene blue in sea water at pH 9.5. Symbol ○ represents the dye taken up by chloroform from methylene blue in sea water at pH 5.5, then freed from chloroform by evaporation, and finally dissolved in distilled water. Symbol ● represents the dye extracted by the sap of *Valonia* from chloroform which had previously been shaken with methylene blue in sea water at pH 5.5. The measurements were made on a layer 1 cm. thick.

maximum at 650 mμ; Fig. 1, symbols ▲ and ×), which readily enters the chloroform because at pH 9.5 it is largely in the form of free base; it readily comes out into sap because the acidity of the sap changes it to dye salt which has a low value of K_w. The reddish violet of the dye in chloroform may indicate the absorption chiefly of azure B in the

form of free base and a smaller amount of azure B in form of salt and methylene blue. With the solution at pH 5.5 the dye obtained from chloroform by evaporation gave a primary absorption maximum at 658 mμ (Fig. 1, symbol ○) and the dye extracted by the sap from the chloroform gave a primary absorption maximum at 660 mμ (Fig. 1, symbol ●), indicating that in both cases there was a mixture of azure B and methylene blue (the former containing slightly less methylene blue than the latter). The color of the dye in chloroform was blue, thus showing that if azure B was absorbed it was in form of salt. The amount of azure B in form of salt and of methylene blue absorbed is less than in the case of azure B in form of free base.

The result obtained at pH 9.5 is in complete agreement with that obtained with living cells of *Valonia* (uninjured). Unfortunately it is not possible to compare the result with that of the living cell in solutions at pH 5.5 since in the latter case penetration is so slow that analysis is not possible at present. But if the present theory be correct we might expect a mixture of these two dyes at pH 5.5 to penetrate the cell but only more slowly than azure B from the solution at pH 9.5.

B. Comparison of Nitella flexilis with the Artificial System.

Previous experiments[9] have shown that from methylene blue solution (French or Merck's medicinal) at pH 9.2 azure B is readily taken up by the vacuole of living cells of *Nitella*. For comparison with the behavior of an artificial system the following experiments were carried out.

The sample of methylene blue (Merck's medicinal) dissolved in buffer solution at pH 9.2 or pH 5.5 gave an absorption curve characteristic of methylene blue (Fig. 2, symbol ■).

The same technique as described under *A* was employed (1) for absorption of dye by chloroform from this sample of methylene blue dissolved in diluted buffer solution at pH 9.2 and pH 5.5, and (2) for the extraction of dye from the chloroform by the artificial sap of *Nitella*. It was not possible to use the real sap

[9] In 1926–27 preliminary statements of these results on *Nitella* were made on page 426 of the paper referred to in foot-note 4, and on page 945 of the paper referred to in foot-note 5. In 1928 these results in detail were reported in the paper referred to in foot-note 6, and in *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 563.

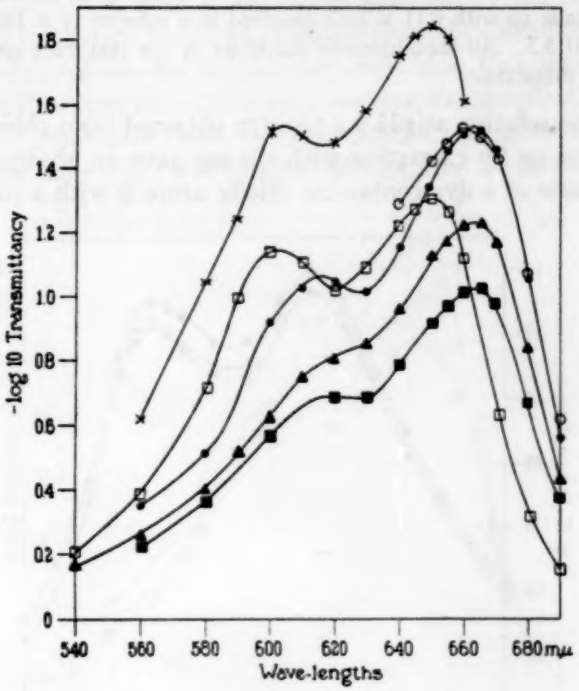

FIG. 2. Absorption curves obtained in experiments on methylene blue solution (Merck's medicinal). (Comparison with *Nitella*.) Symbol ■ represents methylene blue solution at pH 9.2 or at pH 5.5. Symbol □ represents the dye absorbed by chloroform from methylene blue solution at pH 9.2, freed from chloroform by evaporation, and then dissolved in artificial sap of *Nitella*. Symbol × represents the dye extracted by artificial sap of *Nitella* from chloroform which has absorbed the dye from methylene blue solution at pH 9.2. Symbol ○ represents the dye absorbed by chloroform from methylene blue solution at pH 5.5. The chloroform was allowed to evaporate and the dye residue was dissolved in artificial sap of *Nitella*. Symbol ● represents the dye extracted by artificial sap of *Nitella* from chloroform previously shaken with methylene blue solution at pH 5.5. Symbol ▲ represents the dye extracted with distilled water at pH 5.5 from chloroform previously shaken with methylene blue solution at pH 5.5. The curves with the symbols ●, ■, × refer to measurements made with a layer 1 cm. thick, the others to measurements made with a layer 0.3 cm. thick.

of *Nitella* because the protein coagulates on shaking. The artificial sap was therefore made up with 0.11 M KCl dissolved in a solution of M/150 phosphate buffer at pH 5.5. All measurements described in the text were made immediately after extractions.

With the solution at pH 9.2 the dye obtained from chloroform by evaporation or by extraction with the sap gave an absorption curve characteristic of a dye containing chiefly azure B with a primary ab-

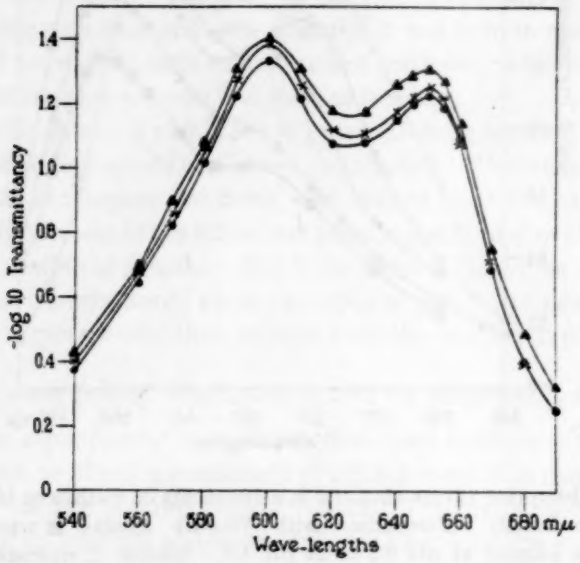

FIG. 3. Absorption curves obtained in experiments on azure B or trimethyl thionine (Holmes) in sea water. (Comparison with *Valonia*.) Symbol × represents the azure B dissolved in sea water at pH 9.5 or pH 5.5. Symbol ● represents the dye extracted by sap of *Valonia* from chloroform shaken with azure B in sea water at pH 9.5; symbol ▲ the same at pH 5.5. The measurements were made on a layer 0.6 cm. thick.

sorption maximum at 650 mμ (Fig. 2, symbol □ and ×). The color of the dye in chloroform was violet red thus indicating that azure B in the form of free base was absorbed. But with the solution at pH 5.5 the dye obtained from chloroform by evaporation gave a primary absorption maximum at 660 mμ (Fig. 2, symbol ○); the dye extracted by the sap from the chloroform gave a primary absorption maximum at 663 mμ (Fig. 2, symbol ●), indicating that a mixture containing chiefly

methylene blue with some azure B was absorbed by the chloroform, but mostly methylene blue was extracted from the chloroform by the sap. The color of the dye in chloroform was blue, thus showing that some azure B in form of salt or methylene blue was absorbed.

When the experiments were repeated at pH 9.2 with methylene blue (French) the same result was obtained as with Merck's medicinal.

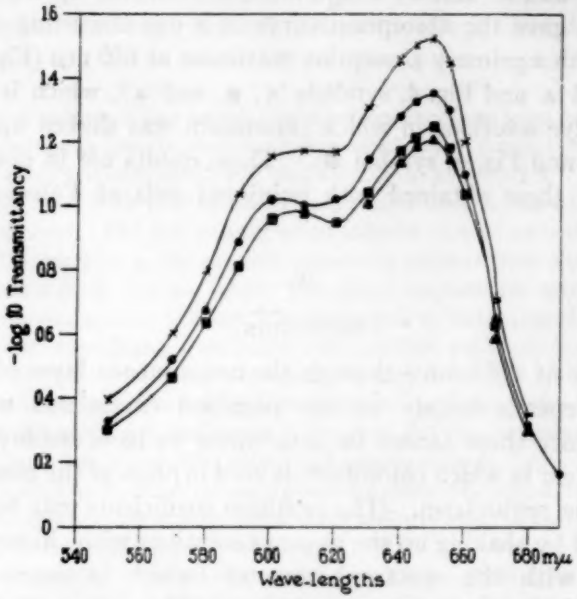

FIG. 4. Absorption curves obtained from experiments on azure B (Holmes) dissolved in buffer solutions. (Comparison with *Nitella*.) Symbol ■ represents the dye dissolved in buffer solution at pH 9.2 or at pH 5.5. Symbol × represents the dye absorbed by chloroform from azure B solution at pH 9.2. The dye was freed from chloroform by evaporation and dissolved in distilled water. Symbol ● represents the dye extracted by artificial sap of *Nitella* from chloroform shaken with azure B solution at pH 9.2; symbol ▲ the same at pH 5.5. The measurements were made on a layer 0.6 cm. thick.

The results obtained with methylene blue at a pH value a little above 9 were in general agreement with the experiments on living cells (uninjured). At pH 5.5, the comparison is not possible since the penetration is so slow that there is not sufficient dye collected for analysis in the sap before there was a possibility of injury to the cell.

III.

Azure B or Trimethyl Thionine.

Using the method described in section II experiments were repeated with azure B for comparison of the artificial system with *Valonia* (Fig. 3) and with *Nitella* (Fig. 4). In both cases the dye absorbed by chloroform and set free by evaporation or extracted by the sap from chloroform gave the absorption curve of a dye consisting chiefly of azure B with a primary absorption maximum at 650 mμ (Fig. 3, symbols ● and ▲ and Fig. 4, symbols ×, ●, and ▲), which is identical with the dye solutions in which chloroform was shaken up (Fig. 3, symbol × and Fig. 4, symbol ■). These results are in exact agreement with those obtained with uninjured cells of *Valonia* and of *Nitella*.

IV.

CONCLUSION.

The rate of diffusion[10] through the non-aqueous layer of the protoplasm depends largely on the partition coefficients mentioned above. Since these cannot be determined we have employed an artificial system in which chloroform is used in place of the non-aqueous layer of the protoplasm. The partition coefficients may be roughly determined by shaking up the aqueous solutions with chloroform and analyzing with the spectrophotometer (which is necessary with methylene blue because we are dealing with mixtures). This will show what dyes may be expected to pass through the protoplasm into the vacuole in case it behaves like the artificial system.

From these results we may conclude that the artificial system and the living cell act almost alike toward methylene blue[11] and azure B, which supports the notion of non-aqueous layers in the protoplasm.

There is a close resemblance between *Valonia* and the artificial system in their behavior toward these dyes at pH 9.5.

[10] *Cf.* Northrop, J. H., *J. Gen. Physiol.*, 1928–29, xii, 435.

[11] Methylene blue solution is generally found to contain azure B as impurity but in too small a concentration to affect the primary absorption maximum which is at about 665 mμ. It is, however, possible to determine roughly by extraction with chloroform the relative amount of azure B present in methylene blue solutions

In the case of *Nitella*, on the other hand, with methylene blue solution at pH 9.2 the sap in the artificial system takes up relatively more azure B (absorption maximum at 650 mμ) than the vacuole of the living cell (655 mμ). But both take up azure B much more rapidly than methylene blue.

A comparison cannot be made between the behavior of the artificial system and that of the living cell at pH 5.5 since in the latter case there arises a question of injury to cells before enough dye is collected in the sap for analysis.

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since azure B in form of free base is absorbed by chloroform from methylene blue solution much more rapidly than methylene blue itself. Azure B may therefore be readily extracted from methylene blue solution at about pH 9. The dye thus extracted is freed from chloroform by evaporation and is dissolved in various aqueous solutions. The dye thus extracted contains various proportions of azure B and methylene blue giving primary absorption maxima from 650 mμ (that of the pure azure B) to 662 mμ (nearly that of purest available methylene blue), depending on the amount of azure B in proportion to methylene blue present in the methylene blue solution from which the extraction was made.

Another method of detection is by observing the color of the dye in chloroform since azure B in form of free base appears violet red, while methylene blue appears blue. But this method is inadequate if the extracted dye is a mixture or if the concentration is low where color matching becomes difficult.

At about pH 9 we find that the more azure B a methylene blue solution contains the more rapid is the rate of penetration of the blue dye into the vacuole of a living cell as well as into the artificial "vacuole."

If the penetration is very slow there are possibilities of errors arising from injury and contamination as already stated (Irwin, M., *J. Gen. Physiol.*, 1928–29, xii, 147) so that the sample "French" was chosen, which had a higher rate of penetration than any other samples (it contained more azure B than others). With *Valonia*, even with the sample "French," the rate of penetration was so slow that it could not very well have been lowered without rendering the results doubtful on account of the possibilities of error already stated. For this reason conclusive results were not possible from experiments with other samples which showed a slower rate of penetration. But with *Nitella* the penetration was more rapid with each of the samples employed so that it was possible to employ a greater variety of samples.

The analogy between chloroform and the living cell is only a rough one. The resemblance, for example, exists in so far as both systems (living and artificial) take up azure B much more rapidly than methylene blue but we need not suppose that the dyes absorbed by both systems will give exactly identical absorption curves in every instance.

These resemblances both in *Valonia* and in *Nitella* become less as injury increases on account of the increase in penetration of methylene blue into the vacuoles of injured cells.

CARBON DIOXIDE FROM THE UNSEVERED VAGUS NERVE OF THE SNAKE.

By G. H. PARKER.

(From the Zoological Laboratory, Harvard University, Cambridge.)

(Accepted for publication, July 3, 1928.)

In all previous tests for the carbon dioxide discharged by nerves these parts have been worked upon after they have been cut free from the animal serving as a source. It therefore seemed desirable to measure the output of this gas from a nerve whose normal relations to the animal were as little disturbed as possible, and whose central and peripheral ends at least were not severed from their connections. After several unsuccessful trials it was decided to attempt this work on the snake's vagus nerve which, in consequence of its considerable length, seemed favorable material for such an investigation. This research was carried out under a grant from the Bache Fund of the National Academy of Sciences. The writer wishes to express his obligation to the Directors of this Fund for the essential aid rendered.

At the outset of the work it was supposed that the vagus nerve of any small snake would serve for the problem at hand, but it was quickly found that even in the largest local snakes the vagus was much too delicate and too short to meet the needs of the experiment. Large snakes were therefore sought, and satisfactory material was finally found in the gopher-snake of Texas and Mexico, *Drymarchon corais couperi* (Holbrook). Snakes of this species were obtained alive from a local collector. They measured in general about two meters in length. They were restrained from undue movement by being placed in a long straight metal tube about 6 cm. in diameter. To manipulate them in the tube, cords were attached one to the snake's jaw and the other to its tail. As a preparatory step the head of the snake was drawn out of the tube for a convenient distance and held there. With a single blow a sharp blade was driven through the head including the brain which was cut through transversely just behind the cerebral

hemispheres thus reducing the animal to the decerebrate condition. An incision was then made along the left-ventral aspect of the body from a point at the base of the skull to the region of the heart. The left vagus nerve was thus laid bare and could be dissected free throughout its exposed length. It remained attached, however,

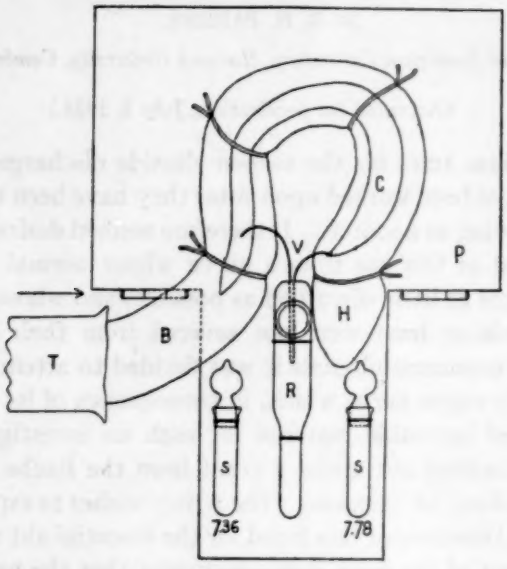

FIG. 1. Plan of the rocking platform and adjacent parts for the support of the anterior portion of the snake. *B*, body of the snake projecting from the metal tube; *C*, cut in the left side of the snake extending from the head to the heart; *H*, head of the snake; *P*, platform; *R*, respiratory chamber with the stoppered aperture uppermost; *S*, standard tubes pH 7.78 and pH 7.36; T, end of the metal tube in which the snake's body was restrained; *V*, vagus nerve looped through a small aperture into the respiratory chamber; its dotted portion corresponds to the outline of the glass rod on which it rests. The arrow on the side marks the axis around which the platform was rocked.

anteriorly to the brain and posteriorly to the region of the heart. In this way from 15 to 20 centimeters of free nerve could be made available.

The anterior portion of the snake over which the cut ran was laid out on a wooden platform (Fig. 1) in such a way as to bring the head and the heart of the animal as near as possible together. The portion

of the snake's body thus made into a loop was tied firmly to the supporting platform and the left vagus nerve already freed was looped in the opposite direction. The free loop of nerve was inserted into the respiratory chamber through an aperture at its end and this aperture was then closed completely by the mixture of kaolin and vasoline already described for this purpose (Parker, 1925, p. 649). Inside the respiratory chamber the loop of nerve rested on a glass rod which ran lengthwise through the larger section of the chamber and close to its axis (Fig. 1).

The respiratory chamber used in these experiments was a tube of pyrex glass much like that employed in my earlier work. It had a capacity of 42 cc. (5 cc. for fluid and 37 cc. for air), and required 0.00686 mgm. of carbon dioxide to change its contents from pH 7.78 to pH 7.36 (Parker, 1925, 1928). The chief differences between the type of chamber used in these tests and that used in the earlier series concerned the apertures. In the present chamber the principal aperture was an opening on the upper side of the tube directly above the central glass rod on which the nerve was placed. Through this opening the nerve could be manipulated and brought into appropriate position. This opening was closed by a tightly fitting ground-glass stopper. In addition to this there were two other openings both at the large end of the chamber. One of these was just above the central glass rod and served for the insertion of the loop of nerve. The other was to one side and was used for the insertion of a capillary glass tube by means of which a current of pure air could be bubbled through the solution in the chamber thus freeing both it and the chamber of carbon dioxide. The two apertures could be closed with the mixture of kaolin and vasoline already described. As in the earlier experiments the respiratory chamber had next to it on the platform a pair of tubes carrying standard indicator solutions one at pH 7.78, the other at pH 7.36.

It usually required from two to two and a half hours to prepare the vagus nerve of a gopher-snake for testing. By the end of this time the period of gush had already passed, if in fact there had been one, and the first possible reading must have represented the steady outflow of carbon dioxide. After the loop of nerve had been put in place the capillary tube was introduced through its special aperture and

sufficient pure air was bubbled through the liquid in the narrow part of the chamber to bring the chamber to a point well on the alkaline side of pH 7.78. The whole platform was then put in rocking motion as already described (Parker, 1925, p. 645). When the color of the indicator in the respiratory chamber agreed with that of the standard tube pH 7.78 a stop-watch was started, and when it later coincided with that in the other standard tube pH 7.36 the watch was stopped.

TABLE I.

Carbon dioxide in milligrams per gram of nerve per minute given out by the vagus nerve of the gopher-snake, *Drymarchon corais couperi* (Holbrook). The snakes are numbered I to VI and their lengths in centimeters are given in the second column of the table. The weight in milligrams of each vagus nerve tested is given in the third column. On the vagus nerve of each snake three or four readings for carbon dioxide were made; these are recorded in the body of the table together with their averages. In each series the last reading was taken after the nerve previously attached anteriorly and posteriorly to the snake had had these attachments severed. Consequently the records to the left of the double vertical line in the table represent records from nerves whose proximal and distal termination were still intact while those to the right of this line represent records taken after these nerves had been cut free.

No.	Length cm.	Wt. mgm.	CO_2 mgm. per gm. per min.				Averages
I	192	127		.0026	.0028	.0025	.00263
II	206	100	.0027	.0020	.0019	.0017	.00208
III	187	96		.0026	.0025	.0022	.00243
IV	198	112	.0031	.0020	.0022	.0018	.00228
V	173	103	.0026	.0026	.0018	.0020	.00225
VI	213	116		.0032	.0023	.0021	.00253
General averages				.00250	.00225	.00205	.00234

The interval of time thus measured represented the period over which the nerve gave out 0.00686 mgm. of carbon dioxide. At the close of an experiment the chamber could be again aspirated with pure air and a second reading taken. In this way it was often possible to get three or four readings from one nerve. At the end of the series the nerve was cut free and weighed. The weight of the nerve and the time required to change the contents of the chamber from pH 7.78 to pH 7.36 having been determined, the rate of discharge of carbon dioxide per gram of nerve per minute could be readily calculated.

Such rates are given in Table I for the vagus nerves of six snakes tested as described. These were the satisfactory trials out of some twenty attempts, the failures being due chiefly to movements of the snake whereby the nerves were dragged out of the respiratory chamber and in some cases torn free at one end or the other. No records were regarded satisfactory unless the heart of the snake was known to have been in continuous action during the test and had been brought to a standstill by an electric stimulus applied to the nerve close to where it emerged from the head of the snake. An inspection of Table I shows that the highest rate of carbon dioxide discharge for this nerve was 0.0032 mgm., the lowest 0.0017 mgm. of gas per gram of nerve per minute. The general average was 0.00234 mgm. This average is very close to that of my earlier determination for the lateral-line nerve of the dogfish, 0.0024 mgm., and only a little higher than that of Fenn (1927) for the same nerve, 0.0020 mgm.

The readings on which the snake determinations were based were taken in the late winter when the snakes were still in a semi-torpid condition. They are therefore more properly compared with the winter rates than with the summer rates for frogs. The average winter rate for the nerves from the large southern frog, *Rana grylio*, was found by me to be 0.0022 mgm., the summer rate 0.0034 mgm. of carbon dioxide per gram of nerve per minute. The agreement in this instance between the winter rate of the frog and of the snake is obvious, but it must be kept in mind that the rates for frog nerves are by no means settled. Fenn (1928) has recorded for the frogs on which he worked relatively high rates, averaging 0.0031 mgm. of carbon dioxide and Gerard (1927) has noted unusually low rates which, if transposed to the terminology of this paper, range from 0.00051 mgm. to 0.00038 mgm. of carbon dioxide per gram of nerve per minute. With Gerard's work carried out at 14°C. and Fenn's and mine at 22°C. it is by no means easy to draw satisfactory comparisons. Subsequent work may settle many of these differences. In the determinations on the snake nerve recorded in this paper all the tests were carried out in a room kept as near 22°C. as possible. It is a remarkable fact that nerves from frogs in the winter and in the summer condition, though tested in a room kept at a reasonably constant temperature, 22°C, nevertheless yield characteristically different results, showing that

notwithstanding the uniformity of the immediate surroundings the metabolic rates of the two classes of nerves are not to be confused.

The chief question in the present research was to determine whether there was a difference in the metabolic rates of nerves whose central and peripheral terminations were intact as contrasted with those in which such connections had been severed. The answer to this question seems to be in the negative. After two or more satisfactory readings had been obtained from the six snake nerves dealt with in this paper and the nerves had been shown by electrical stimulation to be in a condition to check the heart beat and thus to give evidence of normal vitality, they were as a last move cut free from all connections with the animal and a final reading for this state of isolation was taken. Such records are given in the next to the last column of Table I and their average is shown at the bottom of that column. This average, 0.00205 mgm., may be compared with the averages of the two preceding records, namely the immediately preceding one, 0.00225 mgm. and the next in advance of that, 0.00250 mgm. A comparison of these averages shows that the carbon dioxide from these nerves was gradually falling in amount and that this fall was not significantly changed by cutting the central and peripheral connections. In other words, the metabolism of resting nerve is a local activity uninfluenced by central and peripheral connections. It is therefore to be assumed that studies of this kind made on nerve removed from cold-blooded animals are as significant for an understanding of their metabolism as though the work had been carried out on nerves retaining their original connections.

In the instance under consideration it is probable that before the nerves were cut the regular beat of the heart gave evidence that normal nervous impulses were passing over the fibers. Nevertheless, when, by cutting the central and peripheral connections, the passage of these impulses was interrupted no observable change was noted in the carbon dioxide output. That there was such a change is highly probable but the fact that it was not observable probably means that it was a change which as compared with the total metabolism was too small to be noticed by the method here employed. I have estimated (Parker, 1925), that the method that I have used is sensitive to about five per cent of the total metabolism of the tissue studied. Changes that

proceed on less than this proportion would naturally escape detection. The normal activity of the vagus nerve seems to belong to this order and is without doubt well below that degree of activity which is excited when the nerve is artificially stimulated by induction currents. It is probable that in all the results of artificial stimulations as applied to nerve, the production of heat, the absorption of oxygen, and the discharge of carbon dioxide, these records are much in excess of any phase, even the extreme, that is exhibited by nerves under the natural conditions of bodily environment.

SUMMARY.

1. The vagus nerve of the gopher-snake, *Drymarchon corais couperi* (Holbrook), discharges carbon dioxide at an average rate of 0.00234 mgm. of gas per gram of nerve per minute. The extremes were 0.0032 mgm. and 0.0017 mgm.

2. These rates were maintained as well when the nerve was separated from its central and its peripheral connections as when these connections were intact showing that the resting metabolism of nerve is a local operation and is not immediately dependent upon distant connections.

3. These observations also point to the conclusion that the passage of normal impulses over a nerve call for an increase of activity that is unobservable by the method employed and that must be small in amount compared with that produced by artificial stimulation.

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THE PENETRATION OF STRONG ELECTROLYTES.

By W. C. COOPER, JR., M. J. DORCAS, AND W. J. V. OSTERHOUT.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Accepted for publication, June 14, 1928.)

This paper deals primarily with the penetration of strong electrolytes. It touches incidentally on the problem of flotation since in some cases flotation was used as a criterion of penetration: as examples of this we may cite experiments in which NH_4Cl and CsCl were added to sea water.

Striking results were obtained by adding small amounts of 0.6 M NH_4Cl to sea water. The specific gravity of the sea water was very slightly decreased and at first the cells remained at the bottom but after a time they began to float because NH_4Cl penetrated and lowered the specific gravity of the sap so much that the cells rose to the surface: here they continued to live and grow indefinitely. (It is probable that NH_3 or NH_4OH penetrates and is subsequently changed to NH_4Cl[1]).

When the concentration of NH_4Cl in the sea water was 0.005 M[2] enough NH_4Cl penetrated in 10 days[3] to cause the cells to float. Examination showed that the specific gravity of the sap was then lower than that of the sea water (see Table I); it was analyzed to determine the proportions of salts present and it was found that artificial solutions made up in these proportions had practically the same specific gravity as that of the samples of sap.[4] Obviously therefore the penetration of NH_4Cl is sufficient to account for the change in specific gravity (see Table I).

[1] Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1925, xxiv, 234.

[2] This did not increase the osmotic pressure of the sea water more than 0.1 per cent.

[3] These cells were placed in bottles and submerged in the ocean where growth continued.

[4] The composition of these samples will be discussed in a later paper. The specific gravity of the sap in some cases fell (in the course of 22 days) to 1.0195 (taking water at 25°C. as 1.000).

We may surmise that if the ocean contained a little more NH_4Cl many organisms might float which do not now do so. Such a change has great biological importance since it profoundly alters the environment (and the distribution) of an organism to bring it from the bottom to the surface of the water. Some organisms such as *Halicystis*[5] float by excluding certain substances, especially sulfates. It would be exceedingly interesting to know how many mechanisms are employed for flotation and how it can be experimentally produced or suppressed.

Experiments with flotation produced by increasing the specific gravity of the sea water by adding CsCl also gave striking results. When 1 volume of CsCl 0.6 M was added to 9 volumes of sea water the halide content and osmotic pressure remained about the same as

TABLE I.

Specific gravity (at 25°C. taking water at 25°C. as 1.000)		Remarks
Sea water	1.02250	Cells sink
9 volumes sea water + 1 volume CsCl 0.6 M	1.02740	Cells float
Normal sap	1.02250	Cells sink
Sea water containing 0.005 M NH_4Cl	1.02245	Cells sink
Sap of cells which had been 22 days in sea water containing 0.005 M NH_4Cl	[illegible]0	Cells float

in ordinary sea water; the specific gravity rose from 1.0225 (ordinary sea water) to 1.0274. Cells sink[6] in ordinary sea water but after the addition of CsCl they float (see Table I). It is evident that they would sink if sufficient CsCl should penetrate but this did not happen although the cells lived for more than a year: the only cells that sank during this time were a few that died.[7] This experiment recalls that of Loeb[8] on *Fundulus* but differs in that the external medium was not toxic nor of higher osmotic pressure than the sea water.

[5] This was formerly called *Valonia ventricosa*: for an account of its flotation see Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1924–25, vii, 633.

[6] The specific gravity of the sap is normally about the same as that of sea water but the weight of the cellulose wall and of the protoplasm causes the cells to sink.

[7] The cells were kept in finger bowls covered by plates of glass near a north window.

[8] Loeb, J., *Biochem. Z.*, 1912, xlvii, 127.

The experiment was discontinued after 13 months and the sap was extracted by piercing the cells with a sharp glass capillary (after rinsing the outside for a few seconds with distilled water and drying lightly with filter paper). It was found that the halide concentration of the medium (sea water containing CsCl) had risen, as the result of slow evaporation, from 0.564 M to 0.858 M: that of the sap[9] had risen to 0.885 M. The specific gravity of the medium had risen from 1.0274 to 1.0473 and that of the sap[9] to 1.0410.

We are indebted to Professor G. P. Baxter for making a spectroscopic examination of the sap: this showed a very small amount of Cs (probably less than 0.1 per cent); this might have been due to injury of some of the cells, or to contamination of the sap during its extraction. In any case it is evident that little or no Cs penetrated the normal cells during a period of over a year.

A similar experiment was made with RbCl by adding 1 volume of RbCl 0.6 M to 4 volumes of sea water, making the concentration of RbCl 0.12 M. This produced scarcely any change in freezing point depression but raised the specific gravity from 1.0225 to 1.0271. Some of the cells sank after a day and appeared normal but others floated for a few days, after which the experiment was discontinued.

It is therefore apparent that there is little or no penetration of CsCl but it seems probable that there is more penetration or RbCl. We know that KCl and NaCl penetrate (either as such or perhaps as KOH and NaOH)[1] since they are found in the vacuole, but our experiments show that such penetration is very slow.

It would seem that K penetrates most rapidly and Cs least rapidly, the probable order being K > Na > Rb > Cs. Li also penetrates but its position in the series is doubtful. It may be that the alkalies[10] penetrate the protoplasm as undissociated molecules (*e.g.* as hydrates)[11] whose solubility in the protoplasm may increase in the order given. Höber[12] has recently suggested that the order of penetration is that

[9] The normal halide content of the sap is about 0.028 M greater than that of the surrounding sea water and its specific gravity not far from that of sea water.

[10] Nothing is said in this connection regarding NH_4^+ since it probably penetrates to a considerable extent as NH_3.

[11] See Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 234.

[12] Höber, R., and Höber, J., *Arch. ges. Physiol.*, 1928, ccxix, 260.

of the ionic radius but this would require that Cs^+ should enter more readily than K^+.

It should be borne in mind that these ideas cannot be applied generally since in *Halicystis* (formerly called *Valonia ventricosa*[13]) the composition of the sap suggests that Na penetrates more rapidly than K.

It may be of interest to consider the absolute amounts taken up. The best data are those on the absorption of K, Na, and Cl under favorable circumstances. Here, as in other experiments on strong electrolytes, conditions which favor growth favor penetration (as is to be expected since the relation of the salt content of the sap to that of the sea water remains nearly constant). It might be expected that even when no growth occurs cations might penetrate as the result of exchange but our experiments (*e.g.* attempting to exchange K for Na by increasing the K content of the sea water) indicate that such processes must be very slow. Cells placed in bottles and suspended in the ocean (from July 1 to September 5) increased in weight at the rate of about 1 per cent per day.[14] If we take as an average representative of these cells a prolate spheroid 1.3 cm. × 0.78 cm. (this measurement does not include the cell wall) we should have for the surface area of each cell $2\pi\ [b^2 + (ab \div e)\ \sin^{-1}e]$ where a = one half the major axis (= 0.65), b = one half the minor axis (= 0.39), and e, the eccentricity, is 0.8 (from the equation $e^2 = (a^2 - b^2) \div a^2$). This gives for the surface 2.8 sq. cm. The volume is $(4 \div 3)\pi\ ab^2$ = 0.41 cc.

Since the concentration of Cl in the sap is about 0.6 M and the concentration of K[15] is 86.2 per cent of this, 1 liter of sap contains (0.6) × (0.862) = 0.5172 mol of K, and one cell has approximately (0.41 ÷ 1000) 0.52 = 0.000213 mol of K and takes up per day 1 per cent of this or $0.00000213 = 213 \times 10^{-8}$. Since the area of the cell is 2.8 sq. cm. the amount taken up per day per sq. cm. is $(213 \times 10^{-8}) \div 2.8 = 76 \times 10^{-8}$, or $(76 \times 10^{-8}) \div 24 = 3.17 \times 10^{-8}$ mols of K per hour per sq. cm. The intake of Na is this amount multiplied[16] by

[13] Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1924–25, vii, 633.

[14] For measurements of growth see Brooks, M. M., *Am. J. Bot.*, 1925, xii, 617.

[15] This was determined by the perchlorate method.

[16] The molar proportion of Na to K in the sap is 15.1 ÷ 86.2 = 0.17.

0.17 or 0.55 $\times 10^{-8}$ mol of Na per hour per sq. cm.: that of Cl is equal to the sum of K and Na or 3.7×10^{-8}.

It would be interesting to compare these figures with those of Northrop[17] for diffusion through a collodion membrane but it would be necessary to know the pressure which drives K and Na into the cell and we are not able to calculate this with certainty. It may be noted that K moves into the *Valonia* cell against the concentration gradient (the concentration of K is about 40 times as great inside the cell as outside) but Na moves with the concentration gradient (the concentration of Na is nearly 6 times as great outside as inside).

Some experiments with anions may be mentioned here. Cells were placed in a mixture of 3 volumes of sea water + 1 volume of NaI 0.6 M where they floated. After 24 days (under conditions favorable for growth) all the cells still floated except those that had died: the iodide in the sap of the living cells was 0.045 M and in the sea water 0.150 M, but when the growth of the cell is taken into consideration it appears that little or no Cl came out of the cell during the absorption of I.

The case seems to be similar with the intake of Br, at least qualitatively. When 3 volumes 0.6 M NaBr are added to 1 volume of sea water so that the cells float, they sink much sooner when conditions are favorable for growth, indicating that Br is taken up during the process of growth.

A recent paper by Höber and Höber[12] states that considerable bromide penetrates in 7 hours. This is more rapid penetration than occurs in our material unless the cells are injured (we determined Br by the method they employed).

We may conclude that in general the penetration of strong electrolytes into *Valonia* is very slow unless the cells are injured.[18] There

[17] Northrop, J. H., *J. Gen. Physiol.*, 1927–28, xi, 233.

[18] In some cases there is visible temporary injury (as evidenced by the disarrangement of chloroplasts when viewed under the microscope) which is followed by recovery: such experiments are rejected. The best criteria of injury are the microscopic appearance, the turgidity, the absence of disturbance to the protoplasm when the cell is rolled between the fingers or bounced lightly on a table, and the presence of sulfate in the sap. In addition cells should be transferred to sea water and kept under observation but this may tell us nothing in regard to temporary injury during the experiment. *Cf.* Irwin, M., *J. Gen. Physiol.*, 1928–29, xii, 147.

appears to be slow penetration with Li, BrO_3, IO_3, and selenite, but other substances show much less penetration, *e.g.* SCN, ferricyanide, ferrocyanide, formate, salicylate, tungstate, seleniate, NO_2, SO_3, Sb-oxide, glycerophosphate, and many heavy metals and the alkaline earths.

On the other hand our experiments and those of others show that certain weak electrolytes enter *Valonia* more rapidly, *e.g.*, NH_3,[19] H_2S,[20] CO_2,[20] and others.

We might explain these facts on the ground that the protoplasmic surfaces consist of non-aqueous layers[21] through which electrolytes pass for the most part in the form of undissociated molecules. We may imagine that ions striking the outer surface of the protoplasm unite to form molecules and as such pass through the non-aqueous surface layer only to dissociate again on reaching an aqueous phase.[1]

If ions penetrate as such it may be by exchange of those of the same sign going in opposite directions[22] or by the entrance of ion groups (*e.g.* K^+ and OH^-) formed at the surface by collision of ions of opposite sign which then penetrate together. It would seem that such pene-

[19] For experiments on penetration of NH_3 into *Valonia* see Brooks, M. M., *Pub. Health Rep.*, 1923, xxxviii, 2074. For experiments on *Nitella* see Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 235. For experiments on other cells see Warburg, O., *Z. physiol. Chem.*, 1910, lx, 305. Harvey, E. N., *Carnegie Institution of Washington, Pub. No. 183*, 1914, 131. Jacobs, M. H., *The Harvey Lectures*, 1926–27, 146.

[20] Osterhout, W. J. V., *J. Gen. Physiol.*, 1925, viii, 131. Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, ix, 255. For the results of other workers on weak acids see Loeb, J., *Biochem. Z.*, 1909, xv, 254; 1910, xxiii, 95; *Arch. ges. Physiol.*, 1897–98, lxix, 1; 1898, lxxi, 457; Artificial parthenogenesis and fertilization, Chicago, 1913, 143; *J. Gen. Physiol.*, 1922–23, v, 231. Harvey E. N., *Internat. Z. physik.-chem. Biol.*, 1914, i, 463; *Carnegie Institution of Washington, Pub. No. 212*, 1915. Crozier, W. J., *J. Gen. Physiol.*, 1922–23, v, 65, with references to earlier papers. Haas, A. R. C., *J. Biol. Chem.*, 1916, xxvii, 225. Jacobs, M. H., *Am. J. Physiol.*, 1920, li, 321; liii, 457; *Biol. Bull.*, 1922, xlii, 14. Brooks, M. M., *Pub. Health Rep.*, 1923, xxxviii, 1449, 1470. Beerman, H., *J. Exp. Zool.*, 1924–25, xli, 33. Smith, H. W., and Clowes, G. H. A., *Am. J. Physiol.*, 1924, lxviii, 183. Smith, H. W., *Am. J. Physiol.*, 1925, lxxii, 347.

[21] Osterhout, W. J. V., *J. Gen. Physiol.*, 1927–28, xi, 83.

[22] Doubtless the cell can produce a sufficient number of ions for exchange, *e.g.* H^+ and HCO_3^-. *Cf.* Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1928, xxvi, December.

tration must be small in view of the fact that Dr. Blinks finds the electrical resistance of the protoplasm in *Valonia* to be very high. This is so, for example, when the protoplasm is in contact with NH_4Cl although the rise in the pH value of the sap shows that either NH_3 or NH_4OH penetrates rapidly: presumably if NH_4OH penetrates it does so in the form of undissociated molecules.

In the case of strong electrolytes the penetration of undissociated molecules or of ion pairs would probably be much slower than exchange of ions since it could take place only when ions of opposite sign happened to collide at the surface.

SUMMARY.

The entrance of strong electrolytes into *Valonia* is very slow unless the cells are injured. This, together with the very high electrical resistance of the protoplasm, suggests that they may penetrate largely as undissociated molecules formed at the surface of the protoplasm by the collision of ions.

Under favorable circumstances KCl may be absorbed to the extent of 3×10^{-8} mols per hour per sq. cm. of surface together with about 0.17 as much NaCl. Other substances[22] which seem to penetrate to some extent are Li, Rb, Br, BrO_3, I, IO_3, and selenite.

Little or no penetration[23] is shown by SCN, ferricyanide, ferrocyanide, formate, salicylate, tungstate, seleniate, NO_2, SO_3, Sb, glycerophosphate, and many heavy metals and the alkaline earths.

In sea water whose specific gravity had been increased by CsCl cells of *Valonia* floated for over a year and there was little or no penetration of Cs except as the result of injury.

The penetration of NH_4Cl decreases the specific gravity of the sap and causes the cells to float: under these circumstances they live indefinitely. It is probable that NH_3 or NH_4OH penetrates and is subsequently changed to NH_4Cl. It would seem that if the sea contained a little more ammonia this would be a floating organism.

[23] This statement is based on preliminary experiments only.

THE PERMEABILITY OF DRY COLLODION MEMBRANES. II.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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When freshly made collodion membranes are immersed in water before the complete removal of the organic solvents, the membranes are permeable for all substances of small molecular weight. If the membrane is allowed to dry, however, either before or after placing in water, the permeability to electrolytes is lost and the membranes exhibit in general the same type of permeability as living cells (1–3). They furnish therefore an interesting model of the cell in that the permeability of the "wet" membranes is similar to the dead cell while that of the "dry" membranes is similar to the living cell. The mechanism of the permeability of the "wet" membranes was firmly established by the work of Duclaux and Errera (4) who found that the relative rate of flow of various liquids through these membranes was in proportion to the viscosity of the liquids. There seems no doubt therefore that these membranes consist of a network of capillaries through which the solutions pass. Hitchcock (5) and Bjerrum and Manegold (6) have been able to calculate the size of these pores as of the order of magnitude of 10^{-6} cm. As the percentage of water held in the membrane decreases the size of the pores decreases until with membranes containing 5 per cent or less water the rate of flow of water is too small to be measured and the pore size cannot be determined. Collander has found that semipermeability becomes more marked as the percentage of water decreases and assumes as does Michaelis (7) that this selective action of the dry membranes is due to the fact that the pores become too small to allow the passage of large molecules. The impermeability to electrolytes is accounted for by Michaelis (8) as due to the negative charge on the membrane which prevents the passage of negative ions while secondary differences in the rate of passage of the ions are ascribed to

differences in the degree of hydration of the ions. The potential differences observed by Michaelis are accounted for as diffusion potentials caused by the great differences between the mobilities of the anion and cation in the pores of the membrane. An alternative hypothesis is that the pores disappear in the dry membrane and that substances pass by dissolving in and diffusing through the collodion. The essential difference between the two points of view consists in that from the point of view of pores, molecules in solution are surrounded by an atmosphere of the solvent molecules and really move relative to them, the membrane simply serving to alter the area and length of the column of solvent through which the solute can diffuse. The permeability is therefore determined by the diffusion coefficient in the solvent and by the effective diffusion area of the membrane.

From the point of view of solution the molecules are considered to diffuse through the material of the membrane and the solvent in contact with the membrane affects the permeability only indirectly, in that the concentration of the solute in the membrane depends upon the partition coefficient of the substance between the membrane and the solvent. In the case of diffusion of substances in solution the results can be predicted at least qualitatively by either point of view, although there is evidence in favor of the solution idea. The simplest case appears to be that of the passage of gases; and in this case, as was pointed out in a preliminary paper (3), the results are more in accord with the idea of solution. It may be mentioned that there are undoubtedly cases where passage takes place through pores, as in the wet collodion membrane or in unglazed porcelain, while there are also cases such as the passage of solutes through a layer of ether or of gases through rubber (9) or metals where the process is one of solution.

Experimental Procedure.

Preparation of Membranes.—5 cc. of Merck's U. S. P. collodion was placed in a 1.5 × 15 cm. test-tube and rotated mechanically in a horizontal position for 20 minutes. The tubes were then allowed to dry for 3 days. They were then filled with water and the membranes removed, drained and dried for 3 days in a desiccator over H_2SO_4. The membranes could be made thicker or thinner by using more or less collodion. Thinner membranes frequently have imperfections and the thicker ones render the experiments slow.

Attachment of the Membrane.—The glass tube to which the membrane was attached was just large enough so that the membrane could be drawn over it smoothly without wrinkling. The tube was first covered with a thin coat of vacuum stop-cock grease and the membrane slipped on. It was then bound firmly in place with rubber bands.

Determination of the Rate of Passage of Gases.—The determination of the permeability for gases was made in the apparatus shown in Fig. 1. The membrane and glass tube were completely filled with gas by running the gas first into the membrane and then into the outside tube, by appropriate manipulation of the stop-cocks. The volume of gas passing through was read from the movement of

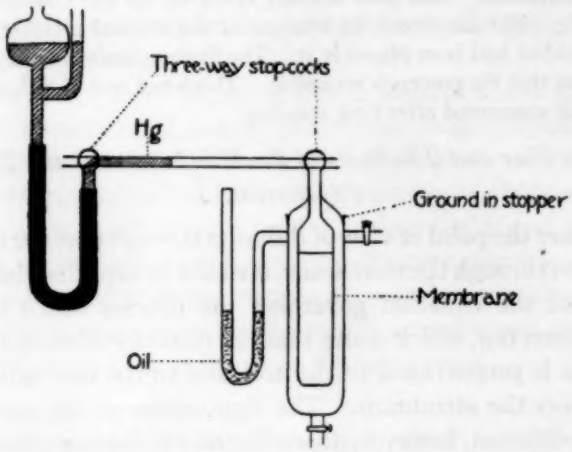

FIG. 1. Apparatus for measuring rate of passage of gases through collodion membranes.

the mercury meniscus and checked by the change in the oil manometer. Since the total volume of the apparatus is large compared to the volume of gas passing through, the oil manometer is very sensitive to changes in barometric pressure and temperature. The entire apparatus was therefore immersed in a large constant temperature water bath. Even under these conditions, unless great precautions are taken against leaks, etc., the change in the outside manometer did not agree quantitatively with that of the inside one.

Determination of the Solubility of Gases.—The volume of gas taken up by collodion was determined in an apparatus essentially the same as that described by Lefebure (10). The apparatus containing the collodion was evacuated to less than a millimeter of pressure for several hours before use.

Determination of the Permeability to Solutes.—A glass tube held in a cork was tied in the end of the membrane and the membrane containing the solution sus-

pended in a test-tube of water. The quantity of solute outside was determined by titration with alkali in the case of acids or by $AgNO_3$ in the case of chlorides. Phenol was determined by titration with iodine.

Determination of the Solubility of Acetic Acid.—Several grams of collodion membrane were placed in a measured volume of the solution and allowed to remain until no further change in concentration of the solution was noted. This required from a day to several weeks depending on the concentration of the solution and the thickness of the membrane. The collodion was then removed from the solution, rinsed with water and placed in a small volume of water. It was placed in fresh water every day until no further solute was found in the water. The wash waters were then combined and the total amount of solute given off from the collodion determined. This total amount taken up by the collodion was also determined by difference, from the analysis of the original solution before and after the collodion had been placed in it. The figures obtained agreed with each other, showing that the process is reversible. This is not true of NH_3 which forms an irreversible compound after long standing.

Effect of the Size and Thickness of the Membrane and the Time of the Experiment.

From either the point of view of diffusion through pores or of solution and diffusion through the membrane, it would be expected that the general form of the equation governing the process would be that of Fick's diffusion law, which states that the quantity of material passing in unit time is proportional to the area and to the concentration difference across the membrane. The significance of the terms of the equation is different, however, depending on which assumption is used.

Assumption I. Diffusion Takes Place through Pores.

If the experiment is arranged so that there is a "steady state," that is, so that the concentration difference across the membrane is constant, the concentration gradient across the (homogeneous) membrane is linear and the equation may be written

$$Q = \frac{P\,t\,A\,(C_1 - C_2)}{h}$$

or

$$P = \frac{Q\,h}{t\,A\,(C_1 - C_2)} \qquad (1)$$

in which Q is the quantity of substance that passes through area A, of thickness h, in time t when the concentration difference in the solution

or gas on the opposite sides of the membrane is $(C_1 - C_2)$. P may be called the permeability constant, since it is the amount of material that will pass through unit area and thickness of membrane in unit time under unit difference of concentration (or pressure). This relation was found to hold both with gases and with substances in solution. The partial pressure of the gas was used. The amount of substance passing at the beginning of the experiment will evidently be smaller than that at the steady state since some of the substance remains in the membrane, so the rate increases slowly at first and then remains constant. The experiments were continued until this constant rate was reached. This required an hour or so for the gases and several days for some of the substances in solution.

Dimensions of P.—It will be noted that if the area and thickness are expressed in the same units and the pressure or concentration in terms of quantity per unit volume, P then has the dimensions: *area* over *time*. Since the mole fraction, which is the significant figure, of the molecular species present in small amount (or the pressure of a gas), is nearly proportional to the mass per unit volume, the concentrations of gases and solutes in low concentration may be expressed as quantity per unit of volume. In these experiments the results are expressed as cm.^{2} per day. The following is an example of the calculation.

A = area membrane = 72 cm^2.
h = thickness membrane = 26×10^{-4} cm. (calculated from area and weight)
t = 2 days
Q = 2.2×10^{-6} mole
$C_1 = \dfrac{10 \times 10^{-6} \text{ mole}}{\text{cm}^3.}$
$C_2 = 0$

$$P = \frac{2.2 \times 10^{-6} \text{ mole} \times 26 \times 10^{-4} \text{ cm.}}{72 \text{ cm.}^2 \times \dfrac{10 \times 10^{-6} \text{ mole}}{\text{cm.}^3} \times 2 \text{ days}} \tag{2}$$

$$= 0.040 \text{ cm.}^2 \text{ per day.}$$

Evidently it makes no difference what units are used to express the quantity and concentration as long as the units are the same in both cases. In the case of a gas, grams or cc. per cm.^{3} at standard pressure and temperature, can be used. In a solution, however, the mole

fraction of the *solvent, i. e.* the molecular species present in excess, is not proportional to its volume concentration, and in this case the dimensions of P cannot be reduced to area over time but must be expressed as quantity per unit membrane size per unit of time and unit of osmotic or vapor pressure, or whatever property of the solvent is assumed to be the determining factor.

From the point of view of pores the value of P is the diffusion coefficient of the substance in water or in whatever substance is supposed to fill the pores of the membrane. It differs from that determined in water without a membrane because it has been expressed per unit area of membrane whereas the area and length of the pores only should have been used. It follows from this point of view that the permeability is a function of the diffusion coefficient of the substance in the solvent and of the effective pore area and length, so that the membrane affects the value only by changing the effective diffusion area. Evidently then the rate of diffusion of the same substance should be different depending on the solvent. A gas for instance should diffuse much more rapidly through a membrane in the gas, in which case the pores are filled with the gas, than through the same membrane immersed in water since the pores would now be filled with water and the rate of diffusion of gases in gases is some 10,000 times as great as the rate of diffusion of gases in water. As will be seen, this is not the case. It would also be predicted that the relative rates of penetration should be in the same order as the diffusion coefficients in water, but the differences should be magnified since the percentage of the total number of pores through which small molecules can pass is greater than that for large molecules. In general this is true, but there are marked exceptions since electrolytes do not pass with appreciable velocity whereas some large molecules, as phenol, pass rapidly. In the case of large molecules having a higher rate of penetration than small ones it is necessary to assume that the diffusion law does not hold in small capillaries or that the substance becomes concentrated in the capillaries.

Assumption II. The Substances Dissolve in and Diffuse through the Material of the Membrane.

The equation for the rate of penetration is the same as before but from this point of view the terms referring to the dimensions of the

membrane are correct but the concentration terms must now refer to the concentration in the two sides of the membrane instead of the concentration in the solution.* If S is the partition coefficient of the substance between the solvent and collodion, *i. e.*

$$S = \frac{\text{Concentration in collodion}}{\text{Concentration in solvent}} \tag{3}$$

then the concentration in the collodion is SC and equation (1) may be written

$$Q = \frac{t\,D\,A\,S\,(C_1 - C_2)\dagger}{h}$$

or

$$D = \frac{Q\,h}{t\,A\,S\,(C_1 - C_2)} \tag{4}$$

$$= \frac{P}{S}$$

D has now the same dimensions as P in the previous case but is of different significance, since it is the diffusion coefficient of the substance in the collodion (more strictly, in a saturated solution of whatever substances are present in the collodion). From this point of view differences in Q, the quantity passing through, are due to differences in the partition coefficient of the substance between the solvent and collo-

* It is assumed, in accord with the work of Noyes and Whitney and others on solution of solids, that equilibrium exists at the interface and that the time element consists in the diffusion. This is a reasonable assumption since the actual interface is of molecular dimensions and the activity of a substance at any point in a solution would differ very slightly from that at another point distant by a few molecular diameters. Unless there were some special block at the interface the activity on the two sides would therefore be expected to be practically identical, *i.e.*, there would always be equilibrium at the interface.

† If the solutions on the two sides are different the partition coefficients will be different and the general equation is

$$Q = \frac{t\,D\,A\,(S_1C_1 - S_2C_2)}{h}$$

This is the case in the experiments reported by Irwin (17).

dion and to the diffusion coefficient of the substance in collodion. This value, D, should therefore depend only on the substance and should be independent of the material surrounding the membrane. Gases therefore should give the same value for D when the membrane is surrounded by gas as when it is in water, and this is the case. The

TABLE I.
Permeability of Collodion Membranes

Substance	H_2	H_2	NH_3	H_2O	N_2	O_2	HCl gas
System measured	$\frac{H_2}{H_2}$	$\frac{H_2}{H_2O}$	$\frac{NH_4OH}{H_2O}$	$\frac{H_2O}{Sugar}$	$\frac{N_2}{N_2}$	$\frac{O_2}{O_2}$	$\frac{HCl}{HCl}$
Moles per liter or pressure at	0.05–1.0	1.0	0.10		0.05–.20	0.05	0.05–.10
P = permeability $= \frac{cm.^2}{day} \times 10^4$	7.5	7.3	1.2		.45	1.1	240
$S = \frac{\text{gm. per cc. collodion}}{\text{gm. per cc. water or gas}}$	.10	.10	2.0	.07	.15	.2	>100
D = Diffusion coefficient in collodion $= \frac{P}{S} = \frac{cm.^2}{day} \times 10^4$	75	73	0.6	30–100	3.0	4.1	2.4?
Molecular weight	2	2	17	18	28	32	36
Molecular radius $\times 10^8$ cm.	1.4	1.4		1.44	1.57	1.6	1.59

values for the permeability should have no general relation to the molecular weight or size except in so far as the partition coefficient between water and collodion varies with these quantities. The values of D should decrease as the size of the molecule increases, although there is no known relation between the diffusion coefficient and the molecular diameter or weight except when the molecule of solute is large compared to that of the solvent. In that case the diffusion coefficient is

inversely proportional to the diameter of the molecule or to the cube root of the molecular weight (11). Empirically it is known that in water the diffusion coefficient of molecules of the same order of size as the water molecule is inversely proportional to the square root of the molecular weight. In the present case the molecules of the solvent,

TABLE I.
...bility of ...ion Membranes.

$\frac{Q_1}{Q_2}$	$\frac{HCl\ gas}{HCl}$	$\frac{HCl}{H_2O}$	$\frac{CO_2}{CO_2}$	$\frac{CO_2}{CO_2\ H_2O}$	$\frac{CO_2}{O_2}$	$\frac{HCOOH}{H_2O}$	$\frac{CH_3COOH}{H_2O}$	$\frac{CH_2NH_2COOH}{H_2O}$	$\frac{C_2H_5OH}{H_2O}$	$\frac{CH_2ClCOOH}{H_2O}$	$\frac{CHCl_2COOH}{H_2O}$	$\frac{HgCl_2}{H_2O}$
0.05-	0.05-.10	0.1	0.05–1	1	1	0.1	0.01–1.0	0.10	0.2	0.1	0.1	0.1
1.1	240	0	6.7	8.0	7.0	.16	.038	0	.15	.025	.0033	.003
2	>100	0	4.6	4.6	4.6	.75	1.0	0	2.8	.36	.11	.16
4.1	2.4?	?	1.4	1.7	1.5	.21	.038	?	.054	.07	.03	.018
32	36		44			46	60	75	90	94	128	270
1.6	1.59		1.61									

collodion, are much larger than those of the solute, and it might be expected that the rate of diffusion would vary as some higher power of the molecular weight.

Experimental Results.

A summary of the results is given in Table I, in which the substances have been arranged in order of their molecular weight. The permea-

bility decreases in an irregular way as the molecular weight increases but there are marked exceptions. HCl gas for instance passes many times more rapidly than hydrogen; and phenol, although of large molecular weight, passes many times more rapidly than acetic acid; while amino acetic does not pass with measurable velocity. The clearest results are those for the gases, since the theory of the passage of gases through fine openings is well worked out and there is less chance for secondary complications. It is known that under all conditions the relative volume of gas passing through a small opening is nearly inversely proportional to the square root of the density of the gas, and that this relation holds even when the capillary is of the order of magnitude of the mean free path of the gas molecule (12). Hydrogen should therefore pass the most rapidly and CO_2 the most slowly. CO_2 and H, however, pass at the same rate while O and N pass much more slowly and HCl goes very much more rapidly. On the other hand, it can hardly be assumed that CO_2 and HCl can pass through pores that will not admit H and the other gases, since the relative diameters of the gas molecules are known and do not differ much, CO_2 being the largest. (There are several independent methods of determining the molecular radius, which lead to different results. The relative order of size, however, is the same for all methods; *cf.* Loeb (12).) On the other hand, when the results are expressed as coefficients of diffusion in collodion, the results are correctly predicted qualitatively for all the substances measured, without any supplementary hypotheses as to electrical effects, etc. Those substances that do not pass the membrane do not dissolve in it, and those like phenol, which are exceptions from the point of view of pores, are found to be soluble. The most striking examples of the effect of solubility, such as NH_3 gas, H_2S gas, and to a certain extent HCl and the collodion solvents of higher molecular weight, cannot be tested from the point of view of solubility since they either destroy the membrane or combine with it irreversibly so that the solubility coefficient cannot be determined. Collodion was found to take up large quantities of NH_3 and H_2S, but the membrane changes color and becomes brittle while only a small part of the gas can be removed. Evidently a chemical reaction takes place subsequent to solution.

TABLE II.

Effect of Water on Passage of H_2 through Collodion.

Membrane factor $= \frac{h}{A} = 2 \times 10^{-5}$.

Membrane	Dried over H_2SO_4		Saturated with H_2O vapor	Immersed in H_2O
Outside	Dry air 1 atmosphere		Air saturated with H_2O 1.1 atmosphere	H_2O, air bubbled through
Inside	Dry H_2 1.1 atmosphere		H_2 saturated with H_2O	H_2 1.1 atmosphere
Partial pressure H — atmosphere	1.1		1.1	1.1
Cc. H_2 per hr	2.2	2.0	2.3	2.05
$P = \frac{\text{cm.}^3}{\text{day}} \times 10^6$	9.6	8.8	10.0	9.0
Order of running experiment	1	4	2	3

The diffusion coefficients of the substances in collodion increase as the molecular weight decreases, which is a reasonable result. The increase, however, is much more rapid than would be expected from the relative rates of diffusion in water, and although no theoretical relation is known between the diffusion coefficient and the molecular weight when the solute molecule is smaller than that of the solvent it appears hardly probably that the diffusion coefficient should increase as rapidly as the figures show. It will be noted, however, that those substances having a molecular weight of 60 and above give diffusion coefficients of about the relative order expected from the molecular weight, while those for O, CO_2 and N are also consistent with each other, as are the values for water and hydrogen. It might be supposed from these results that there are some very small pores and that while the large molecules pass by solution and diffusion the small molecules can also pass through pores. This assumption, however, appears to be ruled out by the fact that H and CO_2 pass at the same rate whether the membrane is immersed in water or in the dry gas.

The result of such an experiment with hydrogen and either a dry membrane or membrane immersed in water is shown in Table II. The rate of passage of the hydrogen is the same within the error of the readings. This is difficult to account for if it is assumed that the hydrogen passes through pores, since, when the membrane is surrounded by gas, the pores must be filled with gas molecules, while when the membrane is in water the gas must diffuse through water, and it is known that the rate of diffusion of gases in water is about 1/10,000 the rate of diffusion of gases in gases. It can hardly be supposed that the pores allow the hydrogen to enter but not the water since the hydrogen molecule is very slightly smaller than the water. Neither can it be assumed that the pressure forced the water out of the pores since the pressure necessary to force water out of such small pores would be many hundreds of atmospheres. From the point of view of solution and diffusion the result is exactly what is expected since the difference in partial pressure of the hydrogen on the two sides of the membrane is 1.1 atmospheres in both cases and the rate of diffusion through the collodion should therefore be the same.

Table III gives the result of a similar experiment with CO_2. In this case the amount of gas passing the membrane was determined by

titration in the outside solution instead of by loss of volume on the inside, so that there is no doubt that the measurement represents the

TABLE III.

Diffusion CO_2, Membrane in H_2O.

Inside	Outside H_2O + M/100 NaOH.			H_2O sat. + CO_2 Time required to bring to pH 8.0 determined		
Membrane No.	2			4		
$F \times 10^5$	2.8			2.8		CO_2/CO_2
Cc. M/100 NaOH	1	1	1	1		
= cc. CO_2	.224			.224	.23	
Δt, hrs.→pH 8.0	.17	.18	.18	.26	.21	
Average	.17			.22		
Cc. CO_2/day/at	31			24		
P = cm.²/day × 10^4	8.6			7.4		6.7

CO_2 diffusion coefficient in H_2O = 1.2 cm.²/day
in O_2 = .18 cm.²/sec. = 1.6 × 10^4 cm.²/day

TABLE IV.

Effect of Water on Permeability of Membrane with Small Holes.

Membrane filled with air under 3 cm. Hg pressure.

8 punctures made in membrane with fine glass needle and rate measured in air and in water.

	Membrane in air	Membrane in H_2O
Cc. per hr.	4000	0.3

TABLE V.

Effect of Area and Method of Preparing Membrane on Quantity of CO_2 Absorbed.

Method of preparing membrane	Poured into water	Membrane made in test-tubes	Membrane made on mercury	
Area membrane per gm. collodion. cm²	?	1100	120	50
Cc. CO_2 per cc. collodion 760 mm. pressure	4.8 4.7 4.3	5.1	4.7	4.7

amount of gas passing through the membrane. Since the radius of the CO_2 molecule is known to be as large or larger than that of water it cannot be assumed that the water was unable to enter the pores.

The experiment was controlled by measuring the rate of diffusion of air through a membrane which had been punctured with a fine glass needle. The result of this experiment is shown in Table IV. The gas now passes more than 10,000 times as fast when the membrane is in air as when it is in water.

The foregoing experiments agree qualitatively at least with the assumption that the passage of substances is governed by solution rather than by diffusion through pores. It is possible, however, to obtain confirmatory evidence from several different experiments.

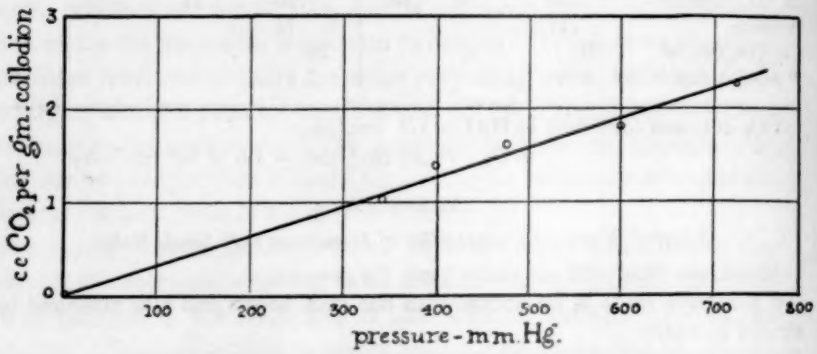

FIG. 2. Solubility of CO_2 in collodion at different pressures.

Effect of Pressure or Concentration and Form of Membrane on the Amount of Substance Dissolved in Collodion.

If the substances form a solution in the collodion it would be expected that the quantity dissolved would be proportional to the concentration or pressure of the substance and independent of the surface of the collodion. Table V shows that the amount of CO_2 absorbed by collodion is independent of the area of the collodion and is nearly the same whether the collodion is prepared by pouring into water or made in the form of a dry membrane. The solubility is a little less in the water sample and it was noted also that some samples of collodion showed a slightly different value for the solubility. Collodion is known to be a mixture and it is possible that the solubility in the various constituents is different, so that different samples would be

expected to vary slightly. If this were the case it might account for the rapid decrease of the diffusion coefficient with increasing molecular weight. But it must be assumed that the membrane is heterogeneous in the plane *perpendicular* to the direction of diffusion.

The amount of CO_2 dissolved by collodion at different pressures is shown in Fig. 2. The quantity is proportional to the pressure up to pressures of 760 mm. Hg.

TABLE VI.

Solubility of Acetic Acid and Phenol in Collodion.

Concentration in H_2O	Concentration in collodion		Partition coefficient = $\frac{\text{Concentration in collodion}}{\text{Concentration in } H_2O}$	
Acetic acid or phenol	Acetic acid	Phenol	Acetic acid	Phenol
mM per cc.	*mM per cc.*	*mM per cc.*		
0.01	0.01		1.0	
.025		0.071		2.8
.05		(.20)		(4.0)
.10	.099	.28	1.0	2.8
.20		.57		2.8
1.0	.87		.87	
5.0	3.05		.61	

TABLE VII.

Solubility of Acetic Acid in Collodion from Acetic Acid-Sodium Acetate Mixtures.

Cc. N/10 HA	10	8	5	2	0
Cc. N/10 NaA	0	2	5	8	10
$\frac{\text{Moles HA per cc. collodion}}{\text{Moles HA per cc. } H_2O}$	.88	.91	1.0	1.0	

The solubility of acetic acid and phenol in solutions of different concentrations in collodion is shown in Table VI. The solubility of acetic acid is proportional to the concentration up to about molar, but above that the concentration of the acetic acid in the collodion increases more slowly than in the solution. The collodion begins to soften at about this concentration of acetic acid and can probably no longer be considered as pure collodion.

Since acetic acid is soluble in collodion while sodium acetate is not, it may be expected that the amount of acetic acid dissolved from a mixture of acetic acid and sodium acetate having the same total acetate concentration would be proportional to the amount of undissociated acetic acid in the mixture. Table VII shows that this is the case.

Independent Solubility of Water and Acetic Acid, etc.

According to the theory of ideal dilute solutions the quantity of substance dissolved should be the same irrespective of the presence of

TABLE VIII.

Weight and Composition of Solution Taken Up.

Solution	Acetic acid		$HgCl_2$	Phenol
	1 M	*5 M*	*.10 M*	*.2 M*
Weight collodion dry gm.	.422	.422	.434	.287
" " $+ H_2O$	.440	.440	.451	.298
" " + solute	.452	.480	.4542	.306
Gm. solute in collodion	.012	.040	.0032	.008
= moles solute $\times 10^5$	20	66	1.2	8.5
Moles per gm. collodion $\times 10^5$	47.5	156	2.7	28
Found by direct titration $\times 10^5$	48	170	2.5	26
Moles per gm. collodion assuming solution of same concentration taken up by collodion	7	14	.46	1.3

Assume adsorption and that each mole HA displaces 1 mole H_2O.

Then total weight per gm. = 170 moles $\times 10^{-5}$ HA + (240–170) $\times 10^{-5}$ moles H_2O

= .1 gm. HA + .012 gm. H_2O

= .112 gm. total

Found .137

other solutes. The amount of water and of various solutes taken up by dry collodion is shown in Table VIII. The results agree with the assumption that the water and solute dissolve independently and cannot be accounted for by supposing that the membrane simply absorbs solution of the same concentration as the bulk of the solution. If it is assumed that the acid is adsorbed in the pores and that each mole of acid displaces one mole of water, the calculation also fails to agree with the experiment. It would be necessary from this point of view

to suppose that each mole of acid displaced much less than one mole of water, or else that the volume of the collodion increased.

Effect of Sodium Chloride on the Permeability to Acetic Acid.

If the acid diffuses through pores in the membrane it might be expected that the presence of concentrated salt solution on the opposite side of the membrane would increase the amount of acid passing through, since the salt forces water to pass through the membrane owing to osmotic pressure and some acid would be carried with the water. On the other hand, the presence of salt in solution with the acid would cause a stream of water to flow toward the acid and might be expected to decrease the amount passing through the pores. There are, however, possible secondary electrical effects or blocks due to partial closing of the pores by salt molecules, so that it is difficult to predict exactly the result of the experiment from this point of view. From the point of view of solubility the presence of the salt on the outside of the membrane should have a negligible effect since the concentration of acid is here practically 0. The presence of salt on the inside should increase the amount of acid passing through, since strong salt solutions increase the activity (vapor pressure) of the acid and should therefore increase the partition coefficient, and hence the permeability. The results of the experiment given in Table IX show that this latter prediction is qualitatively fulfilled but that quantitatively the increased solubility of the acid in the collodion due to the salt is not sufficient to account for the increase in permeability.

Temperature Coefficient.

If the substance diffuses through pores in the membrane the temperature coefficient of permeability should be that found in ordinary diffusion, unless it be further assumed that the pore area of the membrane changes with temperature. If passage takes place by solution almost any temperature coefficient might be expected since the temperature coefficients of solubility are irregular and may even be negative. The temperature coefficient of the *diffusion coefficient* in collodion should be small, however, unless some change occurs in the viscosity of the collodion. Table X gives the results of an experiment in which the rate of penetration of acetic acid was determined at 5°, 25° and 35°C. The

TABLE IX.

Effect of NaCl on Permeability of Acetic Acid.

Inside	M/10 HA H_2O	M/10 HA 5 M NaCl	M/10 HA + 5 M NaCl H_2O	M/10 HA 5 M NaCl
Outside	5 M NaCl solution = 1.2 .1 M HA			
Permeability HA cm.²/day × 10⁴	.028	.029	.042	.027
Diffusion HA in collodion cm.²/day × 10⁴	.028	.028	.035	.027
Partition coefficient HA in collodion from				

coefficient is about 2 for permeability over the whole range, and 1.6 for the diffusion coefficient from 5 to 25, but is the same as that for permeability from 25 to 35.

Permeability to Water.

Membranes made by placing in water before evaporation of the organic solvents may contain more than 5 times as much water as collodion but this water is not in equilibrium since it cannot be replaced once it has been removed without subjecting the membrane to some organic solvent. There is good reason to suppose that this water is held in the pores of the membrane and in fact that the pores are kept

TABLE X.

Permeability of 0.10 Molar Acetic Acid at Various Temperatures.

Temperature	5°		25°		35°
P = cm.²/day × 10^4	0.0086		0.038		0.085
Q_{10}		2.1		2.2	
$S = \frac{\text{Acid per cc. collodion}}{\text{Acid per cc. } H_2O}$	.53		.90		.94
$D \times 10^4 = \frac{P}{S}$ =	.016		.042		.090
Q_{10}		1.6		2.1	

open by the surface tension effects of this water, as Zsigmondy (13) has suggested.

If such membranes are suspended over mixtures of sulfuric acid and water, or even over pure water, they lose weight and finally come to an equilibrium value with respect to water content. This small amount of water is in equilibrium with the membrane and may be removed and replaced repeatedly. The loss of water over pure water is presumably due to the pressure in the collodion network which causes the water to be foreced out in droplets. These droplets have a convex surface and hence a higher vapor pressure than water in bulk. This is a slow process, however, and the loss of weight continues for months. The water content would presumably reach the value obtained by placing dry membranes in water.

The result of such an experiment is shown in Fig. 3 in which the grams of water per gram of collodion are plotted against the vapor pressure of water in the sulfuric acid-water mixtures. These figures are perfectly reversible and are obtained with either the dry membrane or the membrane made by immersing in water before the loss of all the organic solvent. There is some indication, however, that the equilibrium water content of the membranes increases with the original water content. This experiment seems to furnish evidence that water

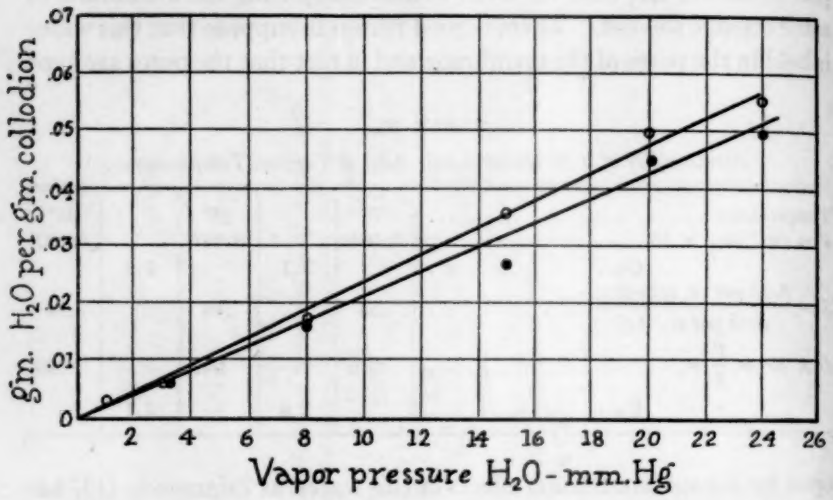

FIG. 3. Water content of collodion membranes over H_2SO_4 water mixtures.
o original water content of membrane 2.70 gm. H_2O per gm. collodion.
● " " " " " .29 " " " " " "

may be held in the membrane in two ways. First, as water held in the capillaries. This may be very large in amount and cannot be replaced once it is removed. Second, a small amount of water is dissolved in the collodion and the amount is proportional to the vapor pressure of water to which the membrane is exposed.

The result may, however, be explained qualitatively from the point of view of pores by assuming that the pores contract until the walls are in contact in places and that the residual water then in the membrane has a different vapor pressure depending on the size of the pore in which

it is held, since it is known that the vapor pressure of a liquid which wets a capillary decreases as the radius of the capillary decreases. From this point of view it is possible then to calculate the radius of these residual pores. The result of this calculation is shown in Table XI. It is evident that the calculated size of the pores is too large, since more than half the water is found to be in pores greater than 1×10^{-7} cm. radius or nearly 100 times the cross-sectional area of the molecules of gas which, from the permeability experiments with gases, are unable to pass through them. Also there is no reason to expect, from this point of view, that the amount of water in the pores should be proportional

TABLE XI.

Radius of Pores from Vapor Pressure (14).

$$ln \frac{P_o}{P} = \frac{2 \sigma M}{RT \delta r}$$

σ = surface tension = 72 ergs/cm.^{2}
M = molecular weight = 18 gm. mole^{-1}
δ = density liquid = 1 gm./cm.^{3}
RT = 2470 $\times$ 10^7 ergs mole^{-1}
r = radius capillary cm.
P_o = vapor pressure liquid in bulk
P = " " " " capillary

$P_o = 24$					
P =	1.2	3	8	15	20
$r \times 10^8$, cm.	3.5	5.0	9.5	22	56
Water in membrane as per cent of maximum	6	10	33	64	90

to the vapor pressure of the water. The calculation, however, includes the doubtful assumption that the angle of contact of the water and collodion is 0. There is also some question as to whether the equation can be applied to such small pores (18).

The permeability of the membranes to water may be determined by filling the membranes with salt or sugar solutions, immersing them in water and determining the increase in weight. Assuming that the water content of the membrane is proportional to the vapor pressure of the solution, the rate of diffusion in the collodion may be calculated. The result of such a calculation is given in Table XII. The vapor

pressure of the solution was calculated from the freezing point depressions in the case of the salt (neglecting the effect of temperature) and assumed proportional to the mole fraction of water in the case of the sugar solutions. It may be noted that the results with the salts although consistent for the various concentrations of the same salt are lower with $CaCl_2$ than with KCl and sugar. This is the reverse of the result obtained by Lucke and McCutcheon (15) with sea urchin eggs.

If the water is assumed to flow through pores then the diameter of the pores may also be calculated from the above data, as was done by Hitchcock (5) and by Bjerrum and Manegold (6).

This calculation is shown in Table XIII. The size obtained in this way is of the order of magnitude of 10^{-9}, or about 100 times smaller

TABLE XII.

Permeability of Membrane to Water When Filled with Various Solutions.

Solution	Sugar		KCl		$CaCl_2$		
	1.0 M	*0.50 M*	*1.0 M*	*0.5 M*	*1.0 M*	*0.5 M*	*0.25 M*
Cc./day/unit membrane × 10^6	1.25	.53	1.2	.60	1.45	.74	.27
Gm. H_2O per cc. collodion water side—gm. H_2O per cc. collodion solution side	.00125	.00062	.00218	.0011	.0033	.00175	.00087
D = cm.²/day × 10^4	100	85	55	55	44	42	31

than from the vapor pressure measurements. It also disagrees with the results of the gas measurements, since the radius is now smaller than the smallest molecular radius. There is again, however, some uncertainty as to the application of the equation to such small pores.

Electroendosmosis.

It might be supposed that the presence of pores could be tested for by electroendosmosis experiments. Since the effect of an electric potential is essentially the same as hydrostatic pressure, it should cause water to pass by diffusion also, and the experiment is therefore inconclusive.

Permeability to Electrolytes.

It has been found by Michaelis and his coworkers that dry collodion membranes act as reversible electrodes for cations but not for anions. That is, in low concentrations of salts a potential approaching the theoretical value of 55 millivolts is obtained when the membrane is placed between 0.10 and 0.01 molar KCl or other univalent neutral salt. These results have been accounted for by Michaelis by the

TABLE XIII.

Calculation of Pore Size and Number from Permeability of H_2O.

Solution	Sugar		KCl		$CaCl_2$		
	1.0 M	*0.5 M*	*1.0 M*	*0.5 M*	*1.0 M*	*0.5 M*	*0.25 M*
Cc. per day per cm.^{2} per cm. thick membrane $\times 10^6$	1.2	.53	1.17	.61	1.45	.74	.27
Osmotic pressure atmospheres	22.4	11.2	39.5	19.5	60.5	31.4	15.7
Cc. per day per atmosphere per unit membrane $\times 10^6$	.056	.048	.029	.031	.024	.023	.017
Radius pore $\times 10^9$ cm.	2.8	2.6	2.0	2.1	1.8	1.8	1.5
No. of pores per cm.$^2 \times 10^{-16}$	1.8	2.0	3.4	3.2	4.2	4.3	5.9

$$r = 1\sqrt{\frac{8\eta Q}{m(w\text{-}1)}}$$

r = radius in cm.
1 = thickness collodion in cm. = 1
η = viscosity of water = .01
Q = cc. water per sec. per dyne
m = gm. collodion per cc. = 1.65
$w = \frac{\text{Wet weight}}{\text{Dry weight}} = 1.04$

$$n = \frac{m^2(w\text{-}1)}{8\pi Q 1^2 \eta}$$

n = no. of pores per cm.^{2}

assumption that the anions are prevented from entering the pores due to an electrical block. The cations can enter the pores but are held back by the attraction of the anions. When a salt solution is present on both sides of the membrane, therefore, the cations can pass through but the anions cannot, so that in the pores the mobility of the cations is much greater than that of the anions. Under these conditions the diffusion potential in the pores should be the same as that expected from a reversible electrode, and this is the case. It would be expected from this point of view that an exchange of cations should take place

when a salt solution is present on both sides of the membrane. Michaelis (16) has found this to be true, but the amount of cations passing through the membrane is exceedingly small. The membranes used in the present experiments gave a concentration potential when placed between 0.10 and 0.01 M KCl of 30 to 40 millivolts. They were impermeable to all electrolytes tried except $HgCl_2$. This salt is more soluble in alcohol than in water and has a measureable solubility in collodion, as might be expected. As noted in Table I it is fairly permeable. About one-third of the membranes tested showed measurable permeability to other electrolytes but the results were irregular and the majority showed no permeability even after a month. It seems necessary to assume that in such experiments the minimum figure is the correct one. The experiments in which the membrane was placed between two salt solutions were also irregular. In the majority of cases no exchange of cations could be noted while in those cases where an exchange occurred there was also some passage of anions. It is possible that this difference from Michaelis' results is due to the different collodion and method of making the membranes. In the case of sodium or potassium acetate separated from HCl, however, there is regularly a passage of the salt through the membrane while no passage occurs when pure water is on the other side of the membrane. The result of such an experiment is shown in Table XIV. It will be noted that both ions of the salt pass through. This result would be expected, however, if the cations could exchange as suggested by Michaelis, since in this case the acetic acid formed can penetrate. If potassium ions passed through, therefore, in exchange with hydrogen ions, acetic acid would be formed in the acetate solution and since the acetic acid could penetrate it would in turn diffuse through and this would continue until the concentration (activity) of the undissociated acetic acid became equal on the two sides of the membrane. The net result would be that both potassium and acetate ions pass through the membrane. From the point of view of solubility these results can be accounted for by assuming that the collodion contains a small amount of an organic acid in solution whose salts are also soluble in collodion. (16). The membrane would therefore act as a reversible electrode for cations as soon as an amount of cation equivalent to the organic acid was taken up since the concentration of the cation in the membrane

would now be constant, a necessary and sufficient condition for a reversible electrode. The low potentials found by Michaelis between concentrated salt solutions would on this basis be due to a very slight independent solubility of the salt itself in the membrane. This explanation has an advantage in that it predicts that the exchange of cations would be very small. As shown in Table XIV this is the case, the penetration of potassium or acetate ion being about 1/10 that for acetic acid. If the impermeability to these ions were due to an electrical block which prevented them from entering the pores, it would be expected when the block was removed by arranging the experiment so that an exchange of ions could take place that the rate of passage of the ions would be of the same order of magnitude as that of molecules of the same size.

TABLE XIV.

Permeability of Potassium Acetate, 30 Days.

Inside		0.10 M. KA		0.10 M. KA	
Outside		.1 M. HCl		H_2O	
Moles Cl^- inside $\times 10^6$		2.0	0		
Moles outside $\times 10^6$	K	6.6	4.0	Trace	Trace
	Acetate	6.0	5.0	"	"
Membrane factor		2×10^{-5}	2×10^{-5}	2×10^{-5}	2×10^{-5}
Permeability $\times 10^7$	Cl	1.0	0		
	K	3.3	2.0	0	0
	A	3.0	2.5	0	0

It is also possible that the membranes contain a few small pores having the properties assumed by Michaelis. The potential developed is independent of the number of pores so that the electrical properties of the membranes may be determined by these occasional and perhaps accidental openings, whereas the actual permeability is determined by solubility and diffusion since if the total pore area is minute the amount of substance passing through them would be negligible.

An essential difference between the two mechanisms exists in the prediction of the results with electrolytes. If the permeability depends on pores and the electrolytes are prevented from passing by an electrical block, then no strong electrolyte can pass. If, however, the permeability depends on solution in the collodion it is quite possible that some electrolytes would have this property and the membrane

would therefore be permeable to them. Mercuric chloride, however, can hardly be cited as an example of this prediction since it is only slightly ionized.

SUMMARY.

The rate of penetration and the solubility of H, O, N, NH_3, H_2O, HCl gas, CO_2, formic, acetic, chloracetic, dichloracetic acid, glycerol, phenol and mercury bichloride in dry collodion membranes have been measured.

The rate of penetration of H and CO_2 is the same whether the membrane and gas are dry or whether the membrane is immersed in water.

The solubility of CO_2, acetic acid, phenol and water in collodion is completely reversible and is proportional to the concentration (or vapor pressure) in low concentrations and independent of the surface of the collodion.

The size of the pores has been calculated from the vapor pressure of water in the collodion and from the rate of flow of water through the membrane. The results do not agree and are not consistent with the observed rates of penetration.

The relative rates of penetration of the gases bear no relation to the density of the gas.

When the results are corrected for the solubility of the substances in the collodion and expressed as the diffusion coefficient in collodion they show that the diffusion coefficient increases rapidly as the molecular weight decreases.

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PHYSIOLOGICAL ONTOGENY.

A. Chicken Embryos.

XIV. The Hydrogen Ion Concentration of the Blood of Chicken Embryos as a Function of Time.

By A. E. COHN and A. E. MIRSKY.

With the Assistance of Yetta Porosowsky.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Accepted for publication, July 13, 1928.)

These experiments continue to illustrate an interest in the processes of growth which are exhibited in earlier communications (1). We have turned now to the study of a more general function and have studied the changes which take place in the hydrogen ion concentration of the blood of chicken embryos. Chickens were selected in the expectation that the new data might form part of the information already accumulated and might serve the purpose of further inferences and wider generalization.

The ages of the embryos studied ranged between 8 and 20 days of incubation. It was impossible to secure sufficient volumes of blood before this age to permit measurements to be made, even when the vessels were successfully punctured by the needle of the syringe. We regret this omission from our data the more because we are aware of the fact that it is precisely at this early age, from 5 to 8 days, that information is desirable. It may be possible at another time to return to this period of the life cycle when methods other than those now available are at our disposal. During the period of hatching and in the first days of life, other difficulties beside that of drawing blood were encountered. Although no difficulty was experienced at the time of hatching in obtaining blood, it was found necessary to carry out this procedure either while the animals struggled or when they had been rendered quiet by the use of anesthetics such as amytal. In both cases, the blood must inevitably be unnatural as the result of dis-

turbance of the oxidation processes, due either to excess lactic acid formation during struggling, or in anesthesia to moderate asphyxia and to diminished rate of blood flow. We learned in the course of our experiments to avoid including measurements taken of the blood of embryos after they had been injured or when they were in a moribund state. In both cases there is a strong tendency for the blood to be more acid than is natural. We came in the end to recognize the presence of these states from examining the blood alone. It has occurred to us that the inclusion of such estimations might result in the publication of erroneous curves.

The incubation of the eggs of White Leghorn hens was arranged in the manner formerly described (2). The ages of the embryos were estimated with care, subject to the difficulties which are usually encountered even when eggs are brought promptly to the incubator after having been laid. The embryos were subsequently studied without removal from the incubator room. The glass electrodes (3), employed in measuring the hydrogen ion concentration, were also installed here. Embryos and their shed blood were, in short, constantly at 38° ± 0.5°C. The wires from the electrodes were led, appropriately sheathed, from the incubator room, through its wall to the adjoining room, where the electrometer was housed.

Blood was drawn either from a vein (arterial blood) or from an artery (venous blood) through a needle into the barrel of a syringe. Into the end of the syringe, a second one which contained the piston was fitted identical with the first. The syringe system from point of needle to piston was filled with paraffin oil. A second person manipulated the piston.

In opening the eggs it was necessary to avoid the occurrence of hemorrhage. Help toward this end was obtained by painting the shell membrane, after enough of the shell was chipped away, with paraffin oil. By this means two objects were attained; first, it was possible to locate blood vessels, and second, capillary hemorrhage was prevented. It is not certain how the effect in preventing hemorrhage took place nor whether the method was free from danger; capillaries may have closed because the membranes, coated with oil, became impermeable to the passage of gases. Usually the amount of the surface of the egg affected in this way was small. When, through

clearing the membrane, blood vessels became visible, it was often possible to insert a needle into them without further dissection—obviously a great advantage. Both oxygenated and reduced blood was obtained from embryos of all ages; in older embryos, both varieties were often drawn from the same animal.

Measurements of the reaction of the blood could be undertaken with volumes no greater than 0.25 cc. When withdrawn the blood

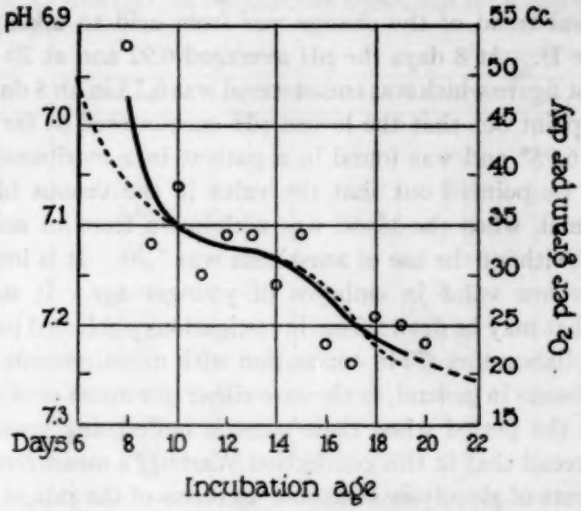

FIG. 1. A curve is shown (the solid line) indicating the course of the change in hydrogen ion concentration in the arterial (reduced) blood of chicken embryos. The circles represent the average pH of the blood at each day of incubation. In the middle of the curve, corresponding to the period between 10 and 14 days, there is a flattening of the curve. A curve (dotted line) published by Murray showing alterations with time of oxygen consumption, flattens at the same period; the ends of the two curves follow similar courses.

was plunged into Ringer solution already under oil in such proportions that the dilution of blood to solution was as 1 to 3 or 4. A small amount of sodium fluoride was likewise present in the test-tube to inhibit glycolysis. The effect of dilution to this extent in the value of the measurement, since it was not sufficiently known, has been studied. Preparations were made of undiluted and of diluted blood and estimated. The difference in value between the two was found

to be no more than a few hundredths of a pH. So diluted and prepared by vigorous stirring to prevent coagulation, the blood was transferred to the cup of the glass electrode. Measurements required so little time that the rate at which they could be made depended on the dissection of the embryos and the withdrawal of blood rather than on the manipulation of the apparatus.

We found a consistent change in the hydrogen ion concentration of the blood throughout that period of embryonic life which we examined. The general trend of the change was from acid to alkaline (Fig. 1 and Table I). At 8 days the pH averaged 6.92 and at 20 days 7.22. The lowest figure which was encountered was 6.75 in an 8 day embryo. We may point out that the lowest pH encountered so far in human beings is 6.95* and was found in a patient in a moribund state (4). It should be pointed out that the value in the venous blood of an adult animal, when the blood was withdrawn from an animal lying quite still without the use of anesthesia was 7.30. It is important to notice the low value in embryos of younger age. It suggests inferences that may be drawn from investigations published by Warburg and his collaborators (5) in connection with measurements of rapidly growing tissues in general, in the case either of tumors or of embryonic tissues in the period when their mass is undergoing great increase. We may recall that in this connection Warburg's measurements dealt with the rate of glycolysis measured in terms of the rate of lactic acid formation. There is to be found in this process not only increase in acid formation but actually an increase in the acid reaction. We are not in position to say that these two phenomena are necessarily mutually dependent. We shall return to this point.

We have for comparison with the results in fowl, studied the blood both arterial and venous, of the fetuses of cats. We were unable, unfortunately, to secure a continuous series so that data could be systematically collected according to time. The fetuses that were studied differed in age but the dating was too uncertain to allow us to draw a curve of the rate of change which takes place. The measurements which were made covered the range from pH 7.04 to 7.31. These it will be perceived lie within that found in chicken embryos. It

* In a personal communication Dr. D. D. Van Slyke tells us that this is the lowest pH of which he is aware.

appears that the reaction which is found in chicken embryos is in no way peculiar to this family, but is characteristic of embryonic and perhaps of rapid growth in general. Okuneff (6) has in point of fact shown that plant tissue in the stage of regeneration after injury likewise exhibits a low pH.

TABLE I.

*The Changes in Hydrogen Ion Concentration of the Blood in Chicken Embryos.**

Incubation age	Reduced blood		Oxygenated blood	
	Average pH	No. of observations	Average pH	No. of observations
days				
8	6.92	13	7.03	8
9	7.12	4	7.18	5
10	7.06	6	7.23	3
11	7.15	4	7.19	1
12	7.11	4		
14	7.16	2	7.19	1
15	7.11	5		
16	7.22	4	7.22	3
17	7.24	3		
18	7.19	5	7.23	4
19	7.20	4	7.20	2
20	7.22	5	7.23	2
Adult	7.30		7.34	

* The standard deviations from the averages are not given. They are wider than would be encountered in the normal resting human subject. At the 8th day for instance, the range is 6.75 to 7.11. We have not given the figures in detail because in our opinion they are in a sense misleading. We believe the wide range is due to the impossibility not only of dating accurately the age of the embryos but also of excluding the possibility of injury. There seems now no reason to doubt that the pH of embryos, if they could be known to be of the same age, would be similar within a narrow range.

On our curve of change in the hydrogen ion concentration, we have inserted the points indicative of the change in oxygen consumption obtained by Murray (7) as expressive of change in the rate of metabolism. It is difficult to escape the impression that the two observe, while not a parallel, at all events a somewhat similar course. As a result of this comparison arises the suggestion either that both are

expressions of the same underlying process, or that they stand in some causal relation to each other. That oxygen consumption and lactic acid formation are both expressions of the metabolic function, is already known. The fact that the curve expressing the change in hydrogen ion concentration takes the form it does in respect to Murray's curve of oxygen utilization raises the question of the possibility of the existence of a relation between lactic acid production and the concentration of hydrogen ions. We are not in possession of measurements which place the formation of lactic acid in a determining position in respect to reaction but have been led to think of this possibility on account of the facts which have just been described.

SUMMARY AND CONCLUSION.

We have studied by means of glass electrodes the hydrogen ion concentration of the blood of chicken embryos from 8 to 20 days. When plotted as a curve, the average data show that a constant change takes place in the measurement, being acid at the beginning of this period and becoming alkaline toward its end. The acid reaction we think was characteristic not only of the embryos of fowl, but of the fetuses of cats and indeed as Warburg has shown of rapidly growing tissue in general. We have suggested the possibility, though we have no data to substantiate the suggestion, that the hydrogen ion concentration may under conditions like these, seeing that the curve of change resembles that of oxygen consumption, be expressive of changes in the rate of metabolism.

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MODIFICATION OF THE NORTHROP-KUNITZ MICROCATAPHORESIS CELL.

By HAROLD A. ABRAMSON.

(From the Laboratory of Research Medicine, Johns Hopkins Medical School, Baltimore, and the Kaiser Wilhelm Institute for Physical Chemistry and Electrochemistry, Berlin-Dahlem, Germany.)

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A modification of the microcataphoresis cell described by Northrop and Kunitz has been used by the author for the study of many different types of suspensions and of different aspects of electrokinetic phenomena. (For a summary and literature review see Abramson: Colloid chemistry symposium monograph, No. VI, New York, 1928.) The essential differences in construction and use of the apparatus as used by the author are briefly described in the following and in Figs. 1 and 2.

1. The cell is not only made entirely of glass, as described by Northrop and Kunitz, but is so blown that the electrode vessels are fused to the cell itself.*

This is of advantage in that:

(*a*) The apparatus is smaller, lighter, more readily handled and is filled with no difficulty.

(*b*) It can be placed directly on the sliding stage of the microscope and can be held in position by two brass extensions which move with the stage.

(*c*) It is easily cleaned with cleaning mixture between measurements with no other manipulation than sliding the entire apparatus out of its support.

(*d*) If it be desired to resuspend particles which have fallen out, the apparatus is simply turned upside down.

2. Instead of Zn-$ZnSO_4$ reversible electrodes it has been found con-

* Two types of glass cells are available: (1) a cell of soft glass of uniform cross-section; (2) a cell of Pyrex or Jena glass drawn out sideways, but not of uniform cross-section. See article in Bibliography for further information.

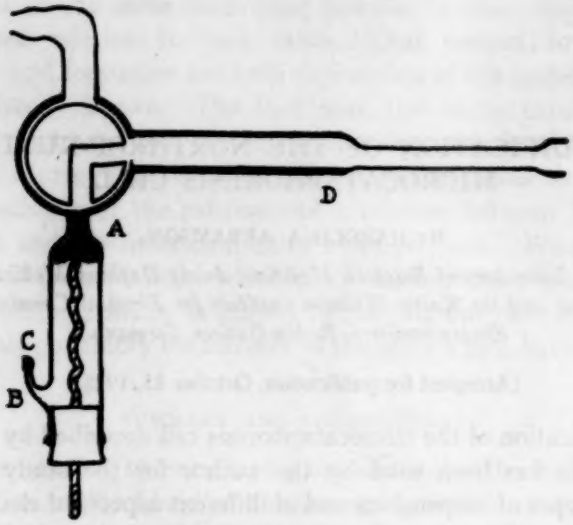

FIG. 1. Schema in longitudinal section of apparatus. At *A* is the agar plug *B* is the outlet, sealed off at *C*. The cell itself is fused to the electrode arm at *D* By reference to previous publications, and Fig. 2, other details can be obtained

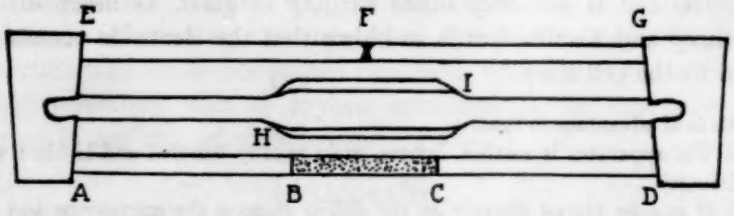

FIG. 2. Schematic view from above. The bars *ABCD* and *EFG* are solid glass supporting rods. Part of the bar *BC* (stippled) is curved so that dark-field illumination from the side is possible. Shaded *F* of the rear supporting rod is a break in *EFG* which is sealed with a suitable cement. By cementing glass plates above and below at the sides *H*, *I* of the cell itself, surfaces are obtained for both manual and mechanical manipulation.

venient to use the following systems, depending on the type of experiment to be made:

(*a*) Cu-$CuSO_4$—electrolyte-free agar

(*b*) Cu-$CuSO_4$—agar in isotonic saline solutions

(*c*) Cu-$CuSO_4$—agar in saturated salt solutions like Na_2SO_4 and KCl.

The use of agar prevents streaming of the liquid from the electrode chamber into the system to be measured. In the case of suspensions in systems with high protein concentrations, as in serum, streaming occurs incidental to the heavy protein precipitate. This streaming, which makes measurement impossible, can be prevented by an agar plug. If the agar plug is electrolyte-free, it is best to allow freshly prepared electrodes to stand overnight before use. Otherwise the applied electrical force may move the agar, slightly, but sufficiently to cause turbulence. Saturated KCl agar has also been used. In the presence of the chloride ion Cu^{++} is apparently reduced by the copper electrode to Cu^{+}. Although the saturated KCl-agar electrode works excellently as a $Cu \rightleftharpoons Cu^{+}$ electrode, a precipitate on the copper may occasionally cause disturbance. Instead of the side arm stop-cocks with a $ZnSO_4$ reservoir as devised by Northrop and Kunitz, the capillary outlets in the diagram are situated as indicated. These facilitate filling the electrode chamber with $CuSO_4$ solution. They are sealed off with a suitable cement after stoppers are in place. A suitable diameter for the electrode vessels is 1 cm. The reproducibility of measurements is considerably enhanced by making consecutive experiments at the same point in the cell. Hairs are crossed and cemented to the top of the cell with Canada balsam. By using the point of crossing of the hairs as a reference mark, it is simple to return to the same point.

3. The calculations of the drop in potential may be accomplished as Northrop and Kunitz suggested. It has been found more convenient to use the following scheme. It follows from Ohm's law that E, the drop in potential per cm. in the cataphoresis cell is

$$E = \frac{IR}{q}, \tag{1}$$

where I is the current, R the specific resistance of the suspension and q the cross-section of the cell at the point of measurement. Since measurement of current prevents errors possible through accidental resistances, it is advisable to use the method of calculation as expressed in equation (1) when possible.

The movements of the water and particle within the cell follow the theory of von Smoluchowski in the case of small E.M.F's. If there is a very high electroendosmotic streaming of the medium, the curve of particle velocity may deviate from the well known parabolic form. This deviation is in the nature of a diminished return flow in the mid-regions of the cell, due perhaps to turbulence phenomena in other parts of the system rather than in the cell itself. Except for these anomalous and mostly avoidable cases, the curve is parabolic. In making measurements one should therefore use the lowest E.M.F. possible for the degree of accuracy required. When the curve of particle velocity at different levels is parabolic, the curve of velocity as plotted against level is the same near the fused ends of the cell itself (within 1.5 mm.) as in the middle. The stream lines of the liquid throughout the cell are therefore uniform. A most useful magnification is that obtained with a Zeiss 28 × ocular and 40 × water immersion objective. The working distance with this objective is 1.6 mm.

SUMMARY.

A modification of the Northrop-Kunitz microcataphoresis cell is described. Although the theory of von Smoluchowski relating to such systems is in general followed, certain deviations may be encountered which are easily avoided.

Addendum.—When the cell is used frequently, it may occur that the current passing through the system gradually falls. This indicates that there is a resistance at some point in the circuit. It can easily be shown that this resistance is at the junction of the agar plug and the solution in the cell itself. I have found that if the agar plug remains in contact with a saturated KCl solution for about 20 minutes, the constancy of the current flow is restored. It seems likely that a thin semi-insulating film of stop-cock grease on the agar surface may be responsible for such erratic behavior of the electrodes.

I am indebted to Professor L. Michaelis and to Professor H. Freundlich for valuable advice received in connection with this investigation.

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STUDIES ON PERMEABILITY OF MEMBRANES.

VII. Conductivity of Electrolytes within the Membrane.

By ARDA A. GREEN, A. A. WEECH, and L. MICHAELIS.

(From the Laboratory of Research Medicine, Medical Clinic, The Johns Hopkins University, Baltimore.)

(Received for publication, July 12, 1928.)

The present communication deals with the results of measurements of the conductivity of the dried collodion membrane and with some accessory experiments which aid in interpreting the conductivity data.

The measurement of the conductivity of a membrane in equilibrium with an electrolyte solution presents certain difficulties which prevent determinations throughout all ranges of concentration and which limit the number of available electrolytes. Under the conditions of measurement the membrane forms only a small fraction of the whole path of the electric current and the conductivity of the membrane can be determined only when the resistance of all other parts of the system is relatively negligible or when the resistance of the other parts can be measured separately. The latter method is certainly the less dependable and is applicable only when the required correction is small. In collecting our data it turned out to be desirable to use two methods of making measurements, each method having its own field of application.

In the first method the ordinary potentiometer outfit used for recording potential differences was utilized. The applied E.M.F. in millivolts necessary to produce a standard deflection of the galvanometer was compared with the E.M.F. required to produce the same deflection when the membrane system was replaced by a known resistance. The membranes used were of the flat type previously described but in which the membrane was fixed to the end of a straight glass cylinder about 1 inch in diameter instead of over a bell jar. A measurement of the resistance was performed with a glass cylinder carrying no membrane, *i.e.*, with a free open end, and then with a membrane

in place, the electrolyte solution being the same in both cases. The difference between the two readings gave the resistance of the membrane. The membrane was kept constantly in the solution being investigated until readings successively constant over several days indicated that equilibrium had been attained. According to the nature of the solution one or several weeks were required for this purpose. The readings obtained under a given condition did not show any drift from the moment at which the electric current was closed, over a period of several minutes. So it is obvious that we are measuring real ohmic resistance and that no appreciable polarizing counterelectromotive force during the time of reading is established which might produce an additional apparent resistance.

This method proved to be suitable for recording the conductivity in solutions of neutral salts such as KCl and LiCl within a range of concentration from 1.0 N to about 0.01 N. In more dilute solutions the resistance of the solution itself was so high as to render correction unsatisfactory. It became evident that the resistance of the membrane was not a constant proportion of the resistance of the whole system. In high concentrations the membrane resistance was many times greater than that of the adjacent solutions. In very dilute solutions the two figures were of the same order of magnitude. On the other hand in the special case of HCl the membrane resistance was so low even in high dilutions as to make the required correction unsatisfactory. The results of these experiments carried out with eight different membranes have been brought together in Fig. I. The relative resistances of the membranes are plotted as ordinates; the abscissæ give the negative logarithm of the concentration. It will be seen that with HCl in all ranges of concentration the resistance is very low, so low that the relatively large corrections involved rob the figures of any absolute meaning. But it is evident in a striking way that the conductivity with HCl is enormously greater than with either KCl or LiCl. In the experiments with these latter salts it will be seen that the resistance with KCl is always much less than that with LiCl, that with both salts the resistance increases as the concentration decreases and that with neither is there proportionality between the resistance and concentration except, perhaps, in solutions more concentrated than $\frac{1}{3}$ normal. The experiments with very dilute solu-

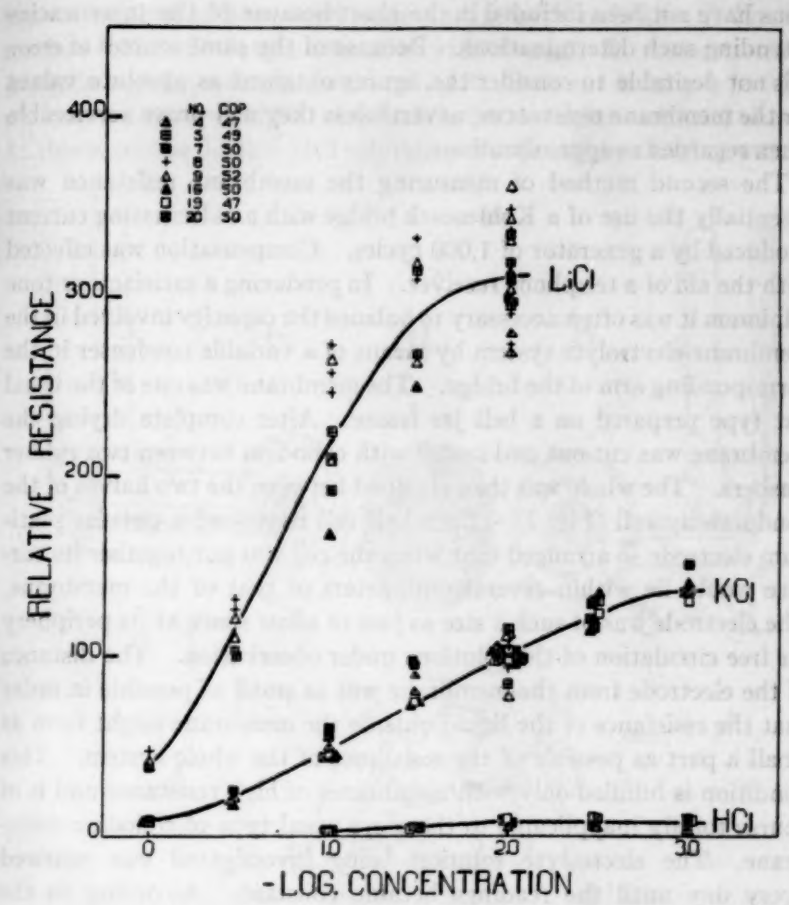

FIG. 1. Showing the relative resistances of the dried collodion membrane in contact with different electrolyte solutions as determined by the first method described in this paper. The experiments from which the figure was drawn were performed with eight different membranes; in most instances more than one determination was made. Since the resistances of these membranes under equal conditions differed widely (25,000–80,000 ohms in 0.01 N KCl) the figures have been brought within the scope of a single chart by setting the average resistance of each membrane in 0.01 N KCl equal to 100 and plotting the points proportionally.

tions have not been included in the chart because of the inaccuracies attending such determinations. Because of the same sources of error it is not desirable to consider the figures obtained as absolute values for the membrane resistances; nevertheless they will prove serviceable when regarded as approximations.

The second method of measuring the membrane resistance was essentially the use of a Kohlrausch bridge with an alternating current produced by a generator of 1,000 cycles. Compensation was effected with the aid of a telephone receiver. In producing a satisfactory tone minimum it was often necessary to balance the capacity involved in the membrane-electrolyte system by means of a variable condenser in the corresponding arm of the bridge. The membrane was one of the usual flat type prepared on a bell jar frame. After complete drying the membrane was cut out and sealed with collodion between two rubber washers. The whole was then clamped between the two halves of the conductivity cell (Fig. 2). Each half cell contained a circular platinum electrode so arranged that when the cell was put together its surface would lie within several millimeters of that of the membrane. The electrode was of such a size as just to allow space at its periphery for free circulation of the solutions under observation. The distance of the electrode from the membrane was as small as possible in order that the resistance of the liquid outside the membrane might form as small a part as possible of the resistance of the whole system. This condition is fulfilled only with membranes of high resistance and is of course totally inapplicable to the more usual type of collodion membrane. The electrolyte solution being investigated was renewed every day until the readings became constant. According to the nature of the solution 3 days to more than a week were necessary for this purpose. The results with any definite solution could be reproduced after some months except for the fact that all resistance data tended to become a little lower as time went on. Obviously the membrane permeability was a little increased in the course of several months. It is surprizing that this deterioration was as slight as it was when the long contact with such solutions as 0.5 N HCl is considered.

This method also proved to be applicable only in limited ranges of concentration. Although no upper limit exists there is a very interesting lower limit. For example, with HCl when in the course of weeks

the concentration is lowered more and more a resistance is finally reached which no longer increases with decrease in concentration. Actually in 0.1 N solution a value of 7,150 ohms was obtained, in 0.01 N solution a value of 22,000 ohms and in 0.001 N solution 26,000 ohms. At this point the 0.001 N HCl solution was replaced by distilled water.

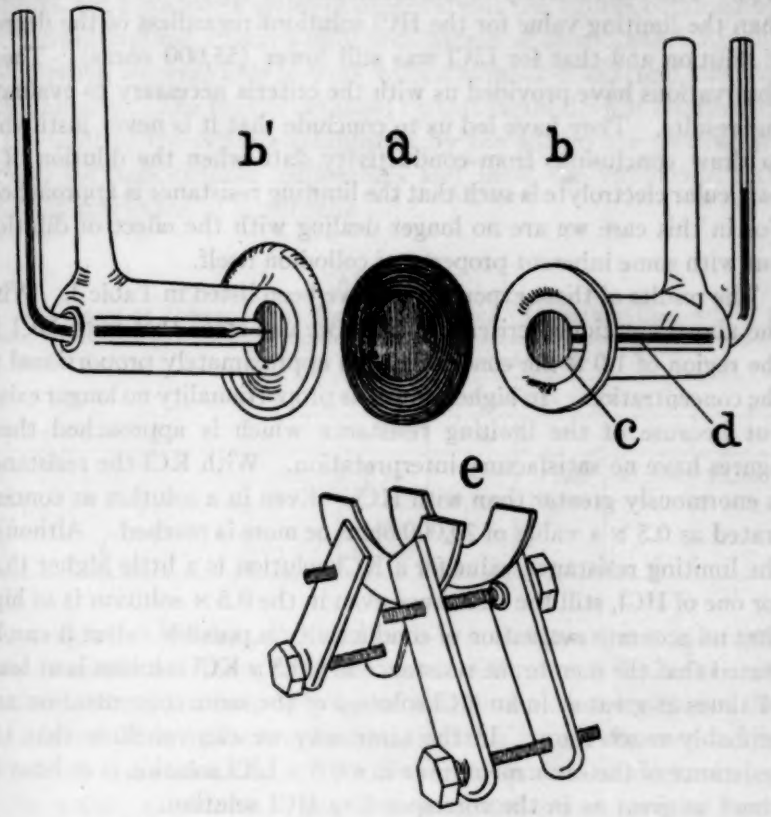

FIG. 2.

An immediate reading of 160,000 ohms was obtained but in the course of some hours this value had decreased to 30,000 ohms. It was then found that no matter how often fresh distilled water was introduced the final resistance of the system after some hours was the same—about 30,000 ohms. The only interpretation possible seemed to be that

some substance liberated by a slow process of disintegration within the membrane was fixing the level of resistance.

Experiments with other electrolytes, KCl, NaCl and LiCl, likewise disclosed a maximum level of resistance but these levels were quite different from each other and all different from that obtained with HCl. The conductivity of the membrane in 0.5 N NaCl was smaller than the limiting value for the HCl solutions regardless of the degree of dilution and that for LiCl was still lower (55,000 ohms). These observations have provided us with the criteria necessary to evaluate our results. They have led us to conclude that it is never justifiable to draw conclusions from conductivity data when the dilution of a particular electrolyte is such that the limiting resistance is approached. For in this case we are no longer dealing with the effect of dilution but with some inherent property of collodion itself.

The results of these experiments have been listed in Table I. With the above mentioned criteria in mind we can state that with HCl in the region of 1.0 N the conductivity is approximately proportional to the concentration. In higher dilutions proportionality no longer exists but because of the limiting resistance which is approached these figures have no satisfactory interpretation. With KCl the resistance is enormously greater than with HCl. Even in a solution as concentrated as 0.5 N a value of 20,000 ohms or more is reached. Although the limiting resistance value for a KCl solution is a little higher than for one of HCl, still the resistance even in the 0.5 N solution is so high that no accurate evaluation of conductivity is possible. But it can be stated that the membrane resistance in a 0.5 N KCl solution is at least 17 times as great as in an HCl solution of the same concentration and probably much more. In the same way we can conclude that the resistance of the same membrane in a 0.5 N LiCl solution is at least 40 times as great as in the corresponding HCl solution.

In reviewing the figures obtained by the two methods just outlined it is evident that the first method yielded little that was not more satisfactorily obtained by the second. In the second method the use of an alternating current assured the absence of even slight polarization effects and the closeness of the platinum electrodes to the membrane surface rendered the resistance of the solution surrounding the membrane negligible as compared with the membrane resistance.

It was thus possible to make measurements with HCl solutions which were not possible by the first method and we became conscious of the limiting resistance values,—values which must have modified the results obtained by the first method. However, because of the cost of the conductivity cell and the length of time (many months) required for a complete series of observations the figures obtained by the second method concern only one sample of membrane. By the first method we were able to work with a number of different samples of membrane. The results obtained by this method when interpreted in the light of the criteria established by the second are quite satisfactory for approximate estimations and show the variations with different specimens of membrane.

TABLE I.

Resistance as Determined by the Second Method (Alternating Current).

	Temperature 23°C.					
	Molar concentration of electrolyte					
	1	1/2	1/4	1/10	1/100	1/1,000
HCl	650	1,240		7,100	22,500	30,000
KCl		22,500	55,000	75,000		
NaCl		41,000				
LiCl		55,000				

A study of all the data obtained leads us to believe that in solutions more concentrated than N/10 HCl, N/4 KCl or N/3 LiCl the conductivity was approximately proportional to the concentration of the electrolyte. It is to be emphasized that this is not the case in the more dilute concentrations. Here, however, the diminishing mobility of the anion, as shown in previous communications, might have altered this proportionality appreciably. Furthermore it is evident that there is an enormous difference in the conductivity of different electrolytes, the conductivity decreasing in the order HCl > KCl > NaCl > LiCl, the differences being many times greater than if no membrane were present.

In attempting to interpret the data obtained we were faced with two questions. (1) Were the differences in conductivity due entirely

to differences in the specific mobilities of the various cations within the membrane? (2) Might not it be that the degree to which an electrolyte could enter the pores of a membrane depended on the nature of the electrolyte? This latter question was especially suggested by the results of our experiments on the diffusion of non-electrolytes. It was here shown that the relatively slow diffusion of a large molecule, such as glucose, when compared with the many times more rapid diffusion of a smaller molecule, such as acetone, was due to the fact that relatively few pores in the membrane were large enough to permit the entry of the larger glucose molecules.

In order to gather data which would enable us to decide between these possibilities it became desirable to determine the actual electrolyte content of membranes after thorough soaking in different electrolyte solutions. After numerous trials the following method was finally adopted. The chlorides of the various cations were selected because of the accuracy with which the electrolyte content could be determined by titration of chlorine. Relatively concentrated solutions (0.5 N) were used in order to obtain a sufficient quantity of electrolyte for satisfactory analysis. A membrane of the ordinary bell jar type was allowed to stand for several days in contact with the particular chloride solution under investigation in order that its pores might become saturated with the electrolyte solution. At the end of this time the membrane and bell jar were thoroughly washed with distilled water to remove all traces of surface solution. We did not believe that washing could remove any appreciable part of the electrolyte within the membrane pores because of the known fact that the diffusion of an electrolyte through these membranes against pure water is extremely slow. This belief was made certain by the analyses which showed the same electrolyte content whether the membrane were in contact with the washing water for 1 or several minutes. The membrane was now placed between two 0.1 N solutions of chloride-free sodium nitrate and an electric current passed by means of platinum electrodes in such a direction that anions would migrate to the interior of the bell jar. During the first minutes of the experiment the only anion carrying current would be Cl^-; as the experiment continued less and less would be carried by Cl^- and more and more by NO_3^-. We therefore interrupted the experiment from time to time, introduced

fresh nitrate solutions and analyzed the solution from the anode compartment for its content of chlorine. This content became rapidly less with succeeding extractions. From the results we could plot a curve, the asymptote of which represented the original electrolyte content of the membrane for the solution being investigated. As an example

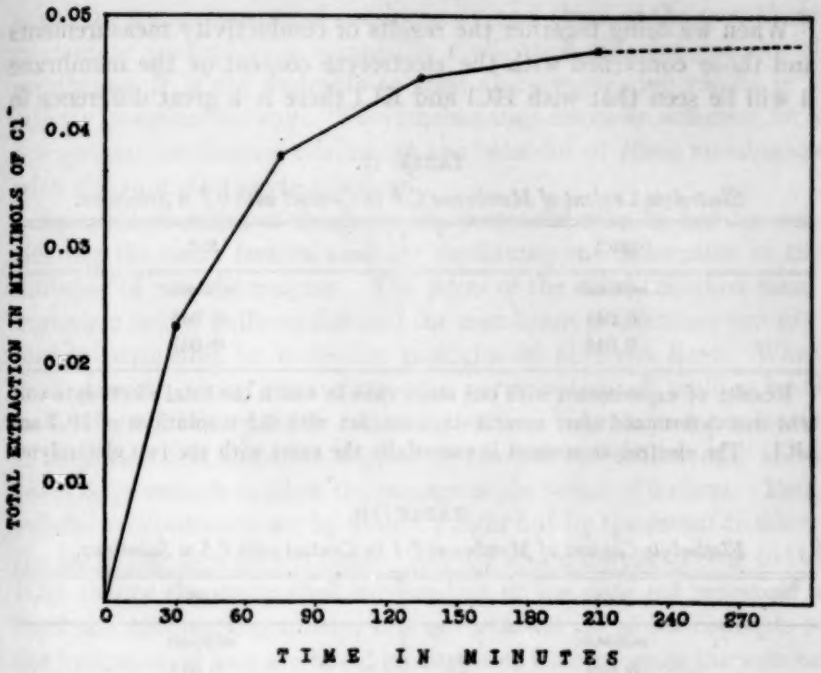

FIG. 3. This chart shows the result of one experiment in which the total electrolyte content of the membrane C-6 was determined after several days contact with a 0.5 N solution of KCl. Repeated extractions of Cl^- were made with a current of constant intensity (4×10^{-3} amperes), the sum of these values being plotted as the total Cl^- extraction in a given time. The asymptote of the curve represents the value of the electrolyte content.

one of these curves obtained with KCl is shown in Fig. 3. Several experiments were made with each solution in order to establish the constancy of the results. Two membranes were used,—one for comparing HCl and KCl, the other for contrasting KCl and LiCl. The results of individual experiments together with the average results

have been listed in Tables II and III. It will be seen that the electrolyte content of a membrane in equilibrium with 0.5 N HCl is approximately the same as with KCl. With LiCl the content is approximately half that with KCl.

DISCUSSION.

When we bring together the results of conductivity measurements and those concerned with the electrolyte content of the membrane it will be seen that with HCl and KCl there is a great difference in

TABLE II.

Electrolyte Content of Membrane C-6 in Contact with 0.5 N Solutions.

HCl	KCl
millimols	*millimols*
0.044	0.045
0.041	0.047

Results of experiments with one membrane in which the total electrolyte content was determined after several days contact with 0.5 N solutions of HCl and LiCl. The electrolyte content is essentially the same with the two electrolytes.

TABLE III.

Electrolyte Content of Membrane I-1 in Contact with 0.5 N Solutions.

KCl	LiCl
millimols	*millimols*
0.019	0.011
0.020	0.011
	0.010

Results of experiments with one membrane in which the total electrolyte content was determined after several days contact with 0.5 N solutions of KCl and LiCl. The electrolyte content of the membrane with KCl is almost the double of that with LiCl.

conductivity whereas the electrolyte content is essentially the same. In this case then we must conclude that the difference in conductivity is due almost entirely to differences in mobility. On the other hand with KCl and LiCl the differences in conductivity correspond fairly

well with the differences in the membrane electrolyte content,—the conductivity and electrolyte content with KCl being roughly the double of that with LiCl. Here it is apparent that no unusual change in mobility need be assumed to explain the result. Inasmuch as for the reasons already given conductivity measurements of the dried collodion membrane do not possess the highest order of accuracy and because the measurements of conductivity and those of the membrane electrolyte content were necessarily made with different samples of membrane, it is not desirable to treat the comparative results in a strictly quantitative way. Nevertheless they are quite sufficient for a few general conclusions concerning the behavior of these membranes with different electrolyte solutions.

An understanding of the facts just presented may be had by considering the same factors used for explaining the differences in the diffusion of non-electrolytes. The pores of the dried collodion membrane are not of uniform size and the membrane is therefore not uniformly permeable to molecular particles of different sizes. When dealing with strong electrolytes the participation of undissociated molecules can be neglected. An anion cannot migrate independently of its cation and therefore a given electrolyte can utilize only those pores large enough to allow the passage of the larger of its ions. Ionic volume is determined not by atomic weight but by the extent to which the particular ions are hydrated. We are aware that according to the Born theory the water shell surrounding an ion does not represent a fixed stoichiometric quantity, but nevertheless all of our concepts of the hydration of ions are based on supposed differences in the volume of the surrounding water shells. We may imagine the sphere of water which sticks so fast to the ion as to resist its separation with a definite force to be different for different kinds of ion. In so far we may speak of differences of volume of the water shell for different ions even though the magnitude of such a volume may not be strictly definable. The results of our experiments are quite in accord with the generally accepted ideas concerning the degree to which the various ions are hydrated. With HCl the H^+ ion is certainly not bigger than the hydrated Cl^- ion and the quantity of HCl entering a membrane is determined solely by the volume of the Cl^- ion. With KCl the volume of the K^+ ion is at least not greater than that of the Cl^- ion and

the latter again determines the quantity of electrolyte which can enter the membrane. In accord with this we found the membrane electrolyte content to be the same with HCl and KCl. On the other hand with LiCl the hydrated Li^+ ion is larger than the hydrated Cl^- ion and it is now the cation which will limit the quantity of electrolyte entering the membrane. In correspondence with this we found the membrane electrolyte content to be much less with LiCl than with either KCl or HCl.

Thus it would seem that two factors are operative in determining the specific conductivity of a membrane in equilibrium with a given electrolyte solution. In the first place the conductivity will depend upon the number of membrane pores large enough to allow the entry of the ions of the electrolyte. But even with different electrolytes which utilize the same membrane pores the ionic mobility and thus the conductivity, because of variations in specific frictional resistance, is much more highly dependent on molecular size than would be true if no membrane were present.

SUMMARY.

Two methods of measuring the electrical conductivity of the dried collodion membrane in contact with an electrolyte solution are described and the results of such measurements with different electrolytes in different ranges of concentration recorded. Some of the difficulties encountered in making these measurements are outlined. Of special interest was the fact that each membrane with each electrolyte showed a maximum level of resistance at a certain point in the dilution scale, a level which was not surpassed by further dilution. It is believed that this level was fixed by the collodion itself rather than by the contiguous electrolyte solution. Its existence limited the results available for reasonable interpretation. In relatively concentrated solutions the conductivity was shown to be approximately proportional to the concentration. With different electrolytes in the same concentration it was shown that the conductivities varied much more than in simple solutions without a membrane and that they fell in the order HCl > KCl > NaCl > LiCl.

A method was described whereby the electrolyte content of a membrane in contact with different chloride solutions could be determined.

It was shown that a membrane saturated with either 0.5 N HCl or 0.5 N KCl had practically the same total electrolyte content whereas the same membrane in contact with 0.5 N LiCl contained only half the quantity. These results were used in interpreting the conductivity data, the evidence presented strongly suggesting that two factors are operative in causing the widely divergent conductivities recorded with different electrolytes. The first factor depended on the quantity of electrolyte which can enter the membrane pores, a quantity dependent on the size of the pores and the volume of the larger of the two hydrated ions of the electrolyte. This factor was the chief one in determining the difference in conductivity between KCl and LiCl. The second factor was concerned with differences in the mobility of the various cations within the membrane brought about by friction between the moving ions and the pore walls. With KCl and HCl the quantity of electrolytes entering the membrane was in each case the same, being determined by the size of the larger Cl^- ion. The widely different conductivity values were explained as due to the changes in the mobility of the two cations within the membrane pores.

STUDIES ON PERMEABILITY OF MEMBRANES.

VIII. The Behavior of the Dried Collodion Membrane toward Bivalent Cations.

By A. A. WEECH and L. MICHAELIS.

(From the Laboratory of Research Medicine, Medical Clinic, The Johns Hopkins University, Baltimore.)

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Our previous studies of the behavior of the dried collodion membrane toward electrolytes have been made chiefly with the monovalent cations, H^+, Li^+, Na^+ and K^+. We have reported the results of electric transfer experiments, conductivity measurements, potentiometric studies and measurements of the total electrolyte content of the membrane pores. During the course of these various studies a few observations were made concerning the behavior of some membranes with bivalent cations, chiefly Ca^{++}. The results of these observations were somewhat different from what we had expected and at the same time we did not feel able to explain them satisfactorily. Now, however, in the light of more fully developed ideas concerning the structure of the membranes the results begin to have a definite meaning and indeed provide additional evidence in favor of these ideas. We shall give first the experimental results and then discusss briefly their significance.

Experimental Results.

1. The Potential Difference of Concentration Chains with the Chlorides of Bivalent Cations.—In measuring the P.D. established across the dried collodion membrane with 0.1 N and 0.01 N solutions of calcium chloride, the results are at first exceedingly inconstant and only after a period of 3 to 4 days, during which time the solutions are frequently renewed, is it possible to obtain readings which are in any way constant. One must also be sure that the calcium chloride used is not contaminated with salts of any of the monovalent cations

as the relatively slight potential effects due to the former might be totally hidden by the effect of the latter. It is advisable to use a system of intermediary bridges such as has been previously described in order that the solutions in direct contact with the membrane may not be contaminated with KCl from the agar bridges. When these precautions are taken it is possible after some days to obtain successive readings which agree within 2 or 3 millivolts. This degree of accuracy is sufficient for our purposes although less than one can obtain with salts of the monovalent cations. The slight fluctuation of the results is due probably in part to the presence of small amounts of impurities, in part to the fact that the much higher resistance of the membrane in calcium chloride solution lowers the sensitivity of the galvanometer and in part to the lowered permeability of the membranes to the Ca^{++} itself, a feature which will be discussed later. In one membrane which had a previously determined *CoP* value of 49 millivolts (P.D. between 0.1 N and 0.01 N KCl solutions) a final result of about −15 millivolts was obtained. The negative sign is used because the more dilute of the solutions was negative instead of positive as in the case of the monovalent cations. This membrane exhibited essentially the same potential difference when placed between 0.1 N and 0.01 N solutions of barium chloride, namely about −14.5 millivolts. In another membrane whose *CoP* value was 46 millivolts the calcium concentration potential was about −13 millivolts. These figures are almost in agreement with that of −18 millivolts reported by Fujita and Michaelis for the same concentration chain without membrane. So the effect of the membrane upon the potential difference appears negligible in this case.

2. *Electric Transfer Experiments.*—The method of performing these experiments has been previously described. When the solutions on the two sides of the membrane are calcium nitrate and sodium chloride the current can be so arranged that the only ions within the membrane are Ca^{++} and Cl^{-}, migrating in opposite directions. In our experiments the chlorine entering the anode compartment was determined by titration with 0.01 N silver nitrate in the presence of potassium chromate as previously described and the calcium entering the cathode chamber estimated by the Kramer-Tisdall blood technique. In our first experiments performed with electrolytes in concentration of

0.02 N we were not able to obtain a satisfactory agreement between the total transfer as calculated from the current passed and that actually found by analysis. For this reason these figures cannot be used for a critical analysis but it was evident in all of the experiments that more of the curent was transported by Cl^- than by Ca^{++}, this result being the reverse of that obtained with the monovalent cations. Here it will be recalled that the transfer number of the anion in all dilutions was less than 0.5 and that the greater the dilution the smaller it became.

The results of our experiments in 0.1 N concentration were more satisfactory because of a relatively good agreement between the total

TABLE I.

Experiment No.	Current intensity	Time	Total transfer by calculation	Chlorine transferred	Calcium transferred	Total transfer by analysis	Transfer number of anion
	milliamperes	*min.*	*milliequivalents*	millimols	*milliequivalents*	*milliequivalents*	$\frac{Cl^-}{\frac{1}{2}Ca^{++} + Cl^-}$
89	2	30	0.0373	0.0190	0.0180	0.0370	0.513
90	2	30	0.0373	0.0170	0.0156	0.0326	0.521
91	2	30	0.0373	0.0187	0.0146	0.0333	0.561
92	2	30	0.0373	0.0210	0.0146	0.0356	0.590
93	2	30	0.0373	0.0218	0.0152	0.0370	0.589

Results of electric transfer experiments with calcium; 0.1 N calcium nitrate in anode compartment; 0.1 N sodium chloride in cathode compartment.

transfers as calculated and as determined by analysis. These results have been listed in Table I. It will be seen that in every instance the transfer number, expressed in equivalents, of chlorine is close to 0.5 and in general tends to be a little greater than this. All of these experiments were performed with a single membrane (C-l) with a *CoP* of value of about 49 millivolts.

3. Conductivity Measurements.—The difficulties of obtaining reliable measurements of the conductivity of the dried collodion membrane and the limited range of concentration which provides figures suitable for reasonable interpretation have beeen discussed in a previous communication. It will be recalled that our comparative experiments were performed in 0.5 N concentration. The membrane resistance in

HCl was at least 15 times less than in KCl, the order of resistances being HCl < KCl < NaCl < LiCl. With $CaCl_2$ an exceptionally high resistance was encountered and the results obtained cannot be regarded as having any absolute meaning. Suffice it to say that the resistance of a membrane in $CaCl_2$ is much greater even than in LiCl.

4. Total Electrolyte Content of Pores.—The method of estimating the total electrolyte content of the membrane pores by making repeated extractions with the electric current has already been described. The results obtained by this method indicated that a membrane has essentially the same electrolyte content when immersed in a solution of KCl as in one of HCl, but that in a solution of the same concentration of LiCl this content is only half as large. The same sample of membrane used in these previously reported experiments was also tested in 0.5 N solution of $CaCl_2$. Here a much lower total electrolyte content was obtained than with any of the chlorides of monovalent cations. It was found that the same membrane in contact with the $CaCl_2$ solution contained only 20 per cent as much chloride ion as when in contact with LiCl and only 10 per cent as much as when wetted with a KCl solution.

DISCUSSION.

By viewing together the several experimental items just outlined it is possible to obtain an understanding of the behavior of the dried collodion membrane toward the calcium ion and probably toward the bivalent ions in general. The exceedingly small electromotive forces arising from concentration chains indicate that in the case of $CaCl_2$ the equivalent mobilities of the anion and cation within the membrane pores are essentially the same. As this is the same condition which exists in a free aqueous solution when no membrane is present we may state that the membrane exerts no effect in altering the relative mobilities of the anion and the cation. The same conclusion may be drawn from the results of the electric transfer experiments in which the transfer number of the anion, expressed in terms of equivalents (not molarity), 0.5 or slightly greater corresponds closely with the slightly negative potential arising in concentration chains. A diffusion chain of M/10 and M/100 $CaCl_2$ should have, on

the assumption of the equivalent mobility of Ca to equalize the one of Cl, an E.M.F. of −18 millivolts (the more dilute solution being negative). The experimentally found value of −14 millivolts in presence of the collodium membrane shows that a statement saying that the equivalent mobilities of Ca and Cl are equal within the membrane, is legitimate to a rather fair degree of accuracy. So the effect of the membrane, as to lower the relative mobility of the anion, vanishes when the cation is bivalent. Here any membrane effect disappears. The question at once arises as to which of the two actions is responsible for the equality. (1) Has the membrane acted so as to decrease the mobility of calcium to the same extent as it decreases the mobility of chlorine in the case of the chlorides of the monovalent cations? If this explanation be the correct one we need suppose no unusual behavior of the anion. (2) In the presence of calcium, is the specific effect of the membrane in retarding the mobility of chlorine abolished? If this were the proper explanation equality of transfer numbers would result because the chloride ion would move faster in the pressence of calcium than in the presence of monovalent cations. The extremely high electrical resistance of a membrane in equilibrium with a solution of calcium chloride, as measured in our conductivity experiments, indicates that the mobilities of both calcium and chlorine are very much decreased within the membrane and that the main action is to decrease the mobility of calcium rather than to increase that of chlorine.

The results of experiments in which the total electrolyte content of a membrane immersed in a solution of $CaCl_2$ was compared with the content when immersed in KCl and LiCl indicate that the main reason for the low mobility of calcium is not that the membrane exerts any specific action on individual cations and decreases their mobility within the pores but that the membrane is highly impermeable to the calcium ion itself. The explanation for this impermeability is undoubtedly the same as that which we have already advanced to explain the slow rate of diffusion of glucose as compared with glycerol and acetone. Only a small fraction of the total pore area is distributed among pores large enough to permit the passage of the calcium ion. This concept as applied to electrolytes has already been elaborated in connection with the discussion of the difference in membrane

conductivities in HCl, KCl, NaCl and LiCl. We pointed out that the volume of an ion must be regarded as including the surrounding water shell and that the number of membrane pores able to transport an electrolyte is determined by that one of its ions which has the greater volume: Inasmuch as the hydrated calcium ion is considerably larger than the hydrated ions of either hydrogen, potassium, chlorine, sodium or lithium it follows not only that the quantity of electrolyte ($CaCl_2$) able to penetrate the membrane will be determined by the volume of the hydrated calcium ion and be independent of the chlorine ion but also that the conductivity and total electrolyte content must be less than with any of the other electrolytes mentioned.

In a previous paper we have given our reasons for believing that a certain range of pore sizes exists throughout which there is a rapid change in the actual mobility of the anion. All experiences have tended to show that when the pores are relatively large in size the specific effect of the membrane in retarding the mobility of anions is abolished or rendered so small that it escapes observation. Now, in the case of calcium chloride it would be seen that the large size of the hydrated calcium ion allows the salt to pass through the membrane only by way of the largest pores and that these pores are too large to exert any appreciable effect on the mobility of the chloride ion. For this reason large potential differences in concentration chains are not observed and electric transfer experiments show that both the anion and the cation have transfer numbers close to 0.5, when expressed in equivalents.

SUMMARY.

A study of the behavior of the dried collodion membrane toward the bivalent calcium ion showed that:

1. There is almost no potential difference established across a membrane separating two calcium chloride solutions of 0.1 and 0.01 N concentrations.

2. The transfer numbers of chlorine and calcium, as measured in electrical transfer experiments, are both close to 0.5.

3. A sample of membrane in equilibrium with a solution of calcium chloride has an extremely high electrical resistance, greater than is

observed with solutions of the chlorides of any of the monovalent cations.

4. The total electrolyte content of a membrane in equilibrium with a solution of calcium chloride was only 20 per cent of that observed when the solution was lithium chloride and 10 per cent of that found when the solution was potassium chloride.

In explaining these various results it is supposed that (1), (2) and (3) are all the result of (4), that is, of the inability of the calcium ion to penetrate any but the largest of the membrane pores. As the total quantity of electrolyte able to penetrate the membrane is very small the electrical conductivity must also be very small. Moreover, the few larger pores that are large enough to transport the hydrated calcium ion are too large to exert any appreciable effect in decreasing the mobility of the anion. Thus the membrane has no effect in modifying the potentials established across concentration chains with $CaCl_2$ and the transfer numbers determined experimentally are what one would expect if no membrane were present.

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